Neural specific knockdown of cytochrome c oxidase and rescue of deleterious phenotypes with alternative oxidase in *Drosophila melanogaster*

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TIIVISTELMÄ

Tutkimuksen tausta ja tavoitteet: Mitokondrion elektroninsiirtoketjun häiriöt aiheuttavat sairauksia, joita kutsutaan yleisesti mitokondriosairauksiksi. Tällä hetkellä näihin sairauksiin ei ole olemassa parannuskeinoa. Tutkimuksessa mitokondriosairauksien molekulaarista patologiaa mallinettiin *Drosophila melanogasterissa* hiljentämällä hermosoluspesifisti sytokromi-c-oksidaasi -geeni. Tutkimuksen tavoitteena oli karakterisoida hermosoluspesifin COX-KD kärpäsen fenotyyppi ja kokeilla, voidaanko vaihtoehtoisen oksidaasin avulla parantaa vahingollisen fenotyypin omaavat kärpäset.

Tutkimusmenetelmät: Sytokromi-c-oksidaasin alayksiköiden geenien hiljentämiseen käytettiin GAL4-UAS -systeemiä. Kahta eri RNAi-konstruktia ekspressoitiin hermosoluspesifisti kolmella eri GAL4 geeniajurilla. Sekä geenien hiljentymisen että vaihtoehtoisen oksidaasin ekspression varmistamiseksi, kärpäsen hermo- ja lihaskudosleikkeitä värjättiin fluerosoivilla vasta-aineilla. COX-KD kärpästen fenotyyppi määritettiin elinkyvyn (lethality) ja elinkyvyn asteittaisen heikkenemisen (survival) perusteella. Samat kokeet toistettiin kärpäsillä, jotka ekspressoivat joko vaihtoehtoista oksidaasia tai Ndi1:tä COX-KD ohella. Lisäksi elav-GAL4 geeniajurien ekspressio kärpäsen eri kehitysasteissa ja eri kudoksissa määritettiin GFP:n avulla.

Tutkimustulokset: Hermosoluspesifi COX-KD aiheutti *Drosophila melanogasterissa* erilaisia fenotyyppejä, muun muassa osittaista tai täydellistä letaalisuutta, liikuntakyvyn puutteita, sekä elinkyvyn heikkenemistä. Fenotyypit olivat voimakkaampia uros kärpäsissä, sekä kärpäsissä, joissa knockdown kohdistui COX alayksikköön IV. Vaihtoehtoisen oksidaasin ekspressio pystyi osittain tai kokonaan parantamaan vahingolliset fenotyypit. Yllättäen Ndi1:n yhtäaikainen ekspressio COX-KD kanssa aiheutti vakavamman fenotyypin kuin pelkkä COX-KD. Tutkimuksessa huomattin myös, että vaihtoehtoista oksidaasia ekspressoitiin lihaskudoksessa. Tarkempi tutkimus osoitti, että elav-GAL4 ajurit ekspressoivat UAS-transgeenejä myös keskiruumiin lihaksissa kotelovaiheessa sekä aikuisissa kärpäsissä.

Johtopäätökset: Uroskärpästen voimakkaamat fenotyypit aiheutuivat todennäköisesti X-kromosomaalisten geenien ekspressiotasojen kompensaatiosta. COX alayksikön IV hiljennyksen aiheuttama fenotyyppi kuvastaa hyvin alayksikön IV tehtävää COX-synteesin aikaisessa vaiheessa. Voimakkaamman fenotyypin takia voidaan olettaa, että alayksikön IV hiljentäminen johtaa vakavampaan sytokromi-c-oksidaasin puutokseen kuin alayksikön Vb hiljentäminen. Vaihtoehtoisen oksidaasin parantava vaikutus perustuu sen kykyyn palauttaa protonien pumppaus kompleksi I:n läpi, aiheuttaen ATP-tuoton nousun tasolle, joka sallii olennaiset tapahtumat soluissa. Ndi1 puolestaan vähentää edelleen protonien pumppausta kompleksi I:n läpi, saaden aikaan päinvastaisen vaikutuksen. Tämän ja edellisten tutkimusten perusteella vaihtoehtoisella oksidaasilla on potentiaalia lääkinnälliseksi molekyyliksi.

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ABSTRACT

Background and aims: Defects in mitochondrial respiratory chain produce diseases commonly referred as mitochondrial diseases. Mitochondrial diseases most often affect tissues that have high energy demands, particularly muscle and nervous system, and they are characterized by a plethora of symptoms. In this study, pathological effects of mitochondrial diseases were simulated in *Drosophila melanogaster* by neural specific cytochrome c oxidase knockdown. The aims of this research were to characterize the phenotype of neural specific COX knockdown in flies and test whether the alternative oxidase (AOX) can rescue the deleterious phenotypes.

Materials and methods: Two UAS-RNAi constructs were used to knockdown COX subunits IV and Vb. RNAi was expressed in neurons by three neuronal GAL4 drivers. To verify COX knockdown in nervous system, sections of paraffin embedded fly muscle and brain were stained with fluorescent antibodies. The expression of AOX was also confirmed using the same method. The phenotype of neuronal COX-KD flies was characterized by i) viability of the flies (lethality assay) and ii) degenerative loss of viability after eclosion (survival assay). The same experiments were conducted on flies that were coexpressing AOX or Ndi1, along with COX knockdown. Additionally, the expression pattern of two elav-GAL4 drivers was determined with GFP in different developmental stages in different tissues.

Results: Neural specific COX-KD using elav-GAL4 drivers presented variable phenotypes, including partial and full lethality, locomotor defects and reduction in viability. The phenotypes were stronger in COX IV-KD flies compared to COX Vb-KD flies and also in males compared to females. AOX expression was able to partially or fully rescue the deleterious phenotypes due to COX-KD. Surprisingly, Ndi1 expression made the phenotypes even worse. AOX was found to be expressed in the thoracic muscles of flies. Closer inspection of the expression pattern of elav-GAL4 drivers revealed that both of them drive the expression of UAS transgenes also in thoracic muscles at pupal and adult stages.

Conclusions: Dosage compensation of X-chromosomal genes can explain the stronger phenotype in male flies. This is true particularly in the case of elav(X)-GAL4. The stronger phenotype in COX IV-KD flies reflects the role of subunit IV in early stages of COX biogenesis. Therefore COX IV-KD is expected to lead to more severe COX dysfunction than COX Vb-KD, which is used in later stage of COX synthesis. AOX was able to fully or partially rescue deleterious phenotypes, which is attributable to its ability to restore proton flow through complex I of the respiratory chain. Thus, ATP production is restored to a level that allows vital cellular functions to operate. Ndi1 expression seems to have the opposite effect. Therefore the level of ATP is expected to be further decreased. This and previous studies show that AOX has true potential to be a therapeutic agent for OXPHOS dysfunction.

CONTENTS

ABBREVIATIONS

AOX = alternative oxidase
ATP = adenosine triphosphate
COX = cytochrome c oxidase
dH_2O = distilled water
elav = embryonic lethal abnormal visual system
GFP = green fluorescent protein
IMM = inner mitochondrial membrane
IMS = intermembrane space
KD = knockdown
mtDNA = mitochondrial DNA
NADH = nicotineamide adenine dinucleotide
Ndi1 = internal NADH dehydrogenase 1
Nrv2 = nervana 2
OMM = outer mitochondrial membrane
OXPHOS = oxidative phosphorylation
RNAi = RNA interference
ROS = reactive oxygen species
TCA cycle = tricarboxylic acid cycle
UAS = upstream activating sequence

1. INTRODUCTION

Mitochondrial diseases are a diverse group of disorders that can affect any tissue in any stage of development and growth. Mitochondrial diseases usually refer to defects of the mitochondrial respiratory chain (DiMauro, 2004). The commonest cause of respiratory chain defect is cytochrome c oxidase deficiency (Schapira, 2006). COX is the terminal enzyme of the respiratory chain and it links the transfer of electrons to proton pumping through the mitochondrial membrane. Currently there is no cure for mitochondrial diseases.

Mitochondrial diseases have been modelled and studied using the fruit fly *Drosophila melanogaster*. Most notably, the knowledge of the full genome and its correspondence to human genome, in addition to several well-established genetical tools, makes *Drosophila* a good model animal for studying diseases (Jacobs *et al*, 2004) . COX deficiencies have also been previously modelled in *D. melanogaster* by knocking down Surf1, a protein involved in COX synthesis (Fernandez-Ayala *et al*, 2009), and COX structural subunits tissue specifically, leading to various phenotypes (Kemppainen *et al*, unpublished). Additionally, affects of COX deficiencies have been modelled in mice (Diaz *et al*, 2006; Li *et al*, 2007), nematodes (Suthammarak *et al*, 2009) and human cells (Antonicka *et al*, 2003).

A possible therapeutic agent for COX deficiencies has emerged through studies of respiratory chain complexes in plants and fungi. These organisms possess alternative respiratory chain enzymes capable of bypassing some of the generic respiratory chain complexes of the mitochondrial OXPHOS system. The alternative oxidase (AOX) can transfer electrons directly from ubiquinone to molecular oxygen, thus bypassing complexes III and IV (Rustin and Jacobs, 2009). After its first discovery in plants and fungi, AOX has also been found in certain lower groups of animals, e.g. in ascidians (McDonald *et al*, 2009) . The therapeutic potential of AOX is based its ability to bypass COX. In COX-deficient patients, the respiratory chain is partially blocked and proton pumping and subsequent ATP production are therefore diminished. If AOX is present, it theoretically will restore electron flow in the first part of the respiratory chain, allowing proton pumping through complex I and partially restoring ATP production (Rustin and Jacobs, 2009).

To test the effect of AOX in COX deficient cells, AOX has been designed to be expressed in various disease models, including *Drosophila* (Fernandez-Ayala *et al*, 2009), human cells (Hakkaart *et al*, 2006) and lately also in mouse (El-Khoury *et al*, 2013). It was shown in these models that

AOX is imported into mitochondria and associated with the inner mitochondrial membrane. It remains inactive in the membrane until the cytochrome component of the respiratory chain becomes blocked. In previous experiments, AOX has been able to restore electron flow in the respiratory chain in human fibroblasts depleted of Cox15 (Dassa *et al*, 2009b) and rescue Parkinson's disease phenotypes and dopaminergic cell loss in *Drosophila* (Fernandez-Ayala *et al*, 2009; Humphrey *et al*, 2012).

In a previous study by Kemppainen *et al* (unpublished), various COX subunits were knocked down in different tissues with RNA interference utilizing the GAL4-UAS system. In the study, neuronal knockdown of subunits COX Vb and COX VIb did not produce any observable phenotypes. It seemed puzzling, why COX deficiency in a tissue with high energy demand would not have any effect. To tackle these questions, the study presented here was performed. The first aim of this study was to characterize the phenotype(s) of neural specific COX knockdown in *Drosophila melanogaster*. The second aim was to test whether AOX can rescue any resulting deleterious phenotypes. COX knockdown was produced by driving the expression of UAS-RNAi constructs against COX subunits IV and Vb with three different neuronal GAL4 drivers. The phenotypes were characterized using lethality and survival assays, which are used to determine the effect of knockdown on survival through development and loss of viability during a two-week testing period following eclosion, respectively. Paraffin sections of fly brain and muscle were stained with fluorescent antibodies in order to evaluate the efficiency and tissue specificity of COX knockdown.

This study provides insights into the pathology of COX deficiencies and also gives information on how AOX is able to alleviate symptoms caused by COX deficiency. With the help of this new information, AOX can be used in future as a therapeutic tool for respiratory chain disorders.

2. REVIEW OF THE LITERATURE

2.1 Mitochondria

Mitochondria are organelles found in all eukaryotes, including plants, algae, animals, fungi and protozoa. They are responsible for most of the ATP production in cells, utilizing the respiratory chain and oxidative phosphorylation for this purpose. Mitochondria are the place for many other metabolic functions as well, for example the citric acid cycle, part of fatty acid metabolism, amino acid metabolism and many other redox reactions. Mitochondria have also other function not related to primary metabolism: steroid hormone synthesis, apoptosis and generation of heat, among others (Alberts *et al*, 2008; Nelson and Cox, 2008). Mitochondria are believed to have originated in evolution through endosymbiosis between an ancient α-proteobacterium and an early eukaryote cell that first happened more than 1.5 billion years ago (Dyall *et al*, 2004). The endosymbiosis theory is strongly supported by the fact that mitochondria harbour their own genome and a protein synthesis machinery that resembles more the bacterial than the eukaryotic cytosolic translation machinery (Alberts *et al*, 2008). Mitochondria are found in all tissues of the human body, and their density reflects the energy need of that particular tissue. Therefore, tissues with high energy need, especially neurons, as well as cardiac and skeletal muscle cells, have a higher density of mitochondria (Schapira, 2006).

2.1.1 Organization and structure

Mitochondria were initially thought of as static organelles, but it is now known that they exist in a dynamic state, in which mitochondria are constantly undergoing fission and fusion (Chan, 2006). The continuously fusing and dividing mitochondria form a mitochondrial network inside the cell (Schapira, 2006). This network is often associated with microtubules, which determines mitochondrial distribution and spacing in different cell-types and tissues depending on their energy requirements (Alberts *et al*, 2008).

Mitochondria are bounded by a double phospholipid membrane, which enclose the intermembrane space between them and the matrix inside the inner mitochondrial membrane (IMM). The outer mitochondrial membrane (OMM) is permeable to small molecules whereas the IMM is impermeable to ions and small molecules alike. Thus the matrix harbours a highly specific content of proteins and other molecules and it is the site of metabolic reactions happening inside mitochondria. The inner mitochondrial membrane is invaginated so that it forms a series of cristae or tubes, protruding into the matrix. Embedded in the IMM are also the enzymes involved in

oxidative phosphorylation and ATP production (Alberts *et al*, 2008). Mitochondria are heavily dependent on nuclear-encoded proteins and enzymes related to transcription, translation and DNA replication and repair, even though they have their own genome and translation machinery (Schapira, 2006). The proteins produced in the cytosol have to be transported into mitochondria to their site of action. Therefore these proteins contain mitochondrial localization signals that reside either in the N-terminal end (most proteins targeted to the matrix and IMM), or internally to the polypeptide (some proteins targeted to IMM). Both mitochondrial membranes have specific protein translocases, designated as the translocase of the outer membrane (TOM) and translocase of inner membrane (TIM), which transport proteins from the cytosol to the intermembrane space and to the matrix, respectively (Neupert, 1997).

2.1.2 Mitochondrial genetics

2.1.2.1 Genome organization, replication, transcription and translation

Mitochondria possess their own genome, which is organized as a double stranded, circular DNA molecule. The genome organization resembles more that of the bacterial than the eukaryotic chromosome, due to the fact that it is not associated with histones. Mitochondrial genomes come in various sizes. The human mitochondrial genome is approximately 16.6 kb long, whereas that of *Drosophila melanogaster* is roughly 19.5 kb (Lewis *et al*, 1995; Alberts *et al*, 2008). The two strands of the mammalian mitochondrial genome are different in their G+T base composition, which makes them separate in caesium chloride density-gradient centrifugation into light and heavy strands. There are many copies of the genome in single mitochondria, and the copy number varies between different types of cells (Taanman, 1999). However, both high and low mitochondrial DNA (mtDNA) copy numbers seem to be associated with elevated risk of developing cancer (Thyagarajan *et al*, 2013; Yu, 2011).

Mitochondrial DNA encodes 22 tRNAs, 2 rRNAs and 13 polypeptides. All of the 13 polypeptides are subunits of the respiratory chain or OXPHOS enzyme complexes. The genes are encoded on both the light and heavy strands. The genes in human mtDNA are very densely packed, meaning there are no introns and only two major non-coding stretches that contain regulatory sites (Taanman, 1999; Fernandez-Silva *et al*, 2003). The *Drosophila* mitochondrial genome harbours the same genes as vertebrates, and they also lack introns. The only distinctive feature of the *Drosophila* mtDNA is the organization and distribution of the genes in the two strands (Berthier *et al*, 1986). In mammals, the major non-coding region in mtDNA is called the displacement loop, or D-loop,

because of its unique triple-stranded structure (Clayton, 2000). The D-loop is adjacent to the origin of replication of the heavy strand and the promoter sites LSP and HSP for the light- and heavy-strands, respectively. The major origin of light-strand replication is located in the shorter non-coding region (only ~30 nucleotides long), situated inside a tRNA cluster (Fernandez-Silva *et al*, 2003). MtDNA is replicated independently of the state of the cell, even in non-dividing cells (Schapira, 2006). Mitochondria possess their own DNA polymerase, polymerase γ that also has a proofreading activity for maintaining sequence fidelity. The mtDNA replication machinery also includes a mitochondrial helicase, Twinkle, that unwinds double-stranded DNA ahead of the polymerase, as well as a mitochondrial single-strand binding protein (mtSSB) that protects single-stranded DNA (Fernandez-Silva *et al*, 2003). The way in which mtDNA is replicated is still under debate. Currently there are three models for mtDNA replication: the strand displacement model, the strand-coupled model and RNA incorporated throughout the lagging strand (RITOLS) model (Kasiviswanathan *et al*, 2012).

As previously mentioned, transcription of mtDNA also initiates from a region adjacent to the D-loop, which contains the promoter sites for both light- and heavy-strand transcription. In fact, there are three initiation sites for transcription because heavy-strand transcription can start from two alternative locations designated as H_1 and H_2. The gene content of the primary transcript depends on the heavy strand initiation point. If transcription starts from H_1, the primary transcript includes genes for the two rRNAs and two tRNAs. If it starts at H_2, one polycistronic primary transcript covering almost the whole heavy strand is formed, including genes for the 12 polypeptides encoded in the heavy strand and 14 tRNAs. Transcription initiation from the light strand promoter yields one polycistronic transcript, including 8 tRNAs and the ND6 mRNA (Fernandez-Silva *et al*, 2003). The mitochondrial transcription machinery includes the mitochondrial RNA polymerase and transcription factors mtTFA and TFB2M, which take part in initiation, and MTERF, which participates in termination of transcription (Taanman, 1999). The polycistronic primary transcripts are cut at both ends of the tRNA genes that flank the rRNAs and the mRNAs by various endonucleases, in order to produce individual rRNAs and mRNAs. These transcripts are then further processed enzymatically (Fernandez-Silva *et al*, 2003). The matured mitochondrial mRNA transcripts bear a 3´-poly A-tail like nuclear transcripts, but they lack a 5´-cap structure (Alberts *et al*, 2008).

Mitochondrial ribosomes, or mitoribosomes, are composed of two mitochondrial rRNAs, 12S and 16S, and around 81 mitoribosomal proteins. The mitoribosomes differ from their cytosolic

counterparts and also from bacterial ribosomes in having significantly less rRNA. Mitochondrial translation includes initiation, elongation and termination steps that are effected by specific translation factors (e.g. mtIF2 and mtIF3 for initiation, mtEFTu and mtEFTs for elongation and mtRF1 and mtRRF for termination). Mitochondria use a genetic code that differs from the universal one in several aspects (e.g. UGA is a universal termination codon, whereas in mitochondria it codes for tryptophan). The mitochondrial code also differs between mammals and *Drosophila*. In addition, mitochondria use a simplified decoding system that allows them to synthesise the 13 polypeptides by using only 22 tRNAs. Mammalian mitochondria use only a single $tRNA^{Met}$ for both initiation and elongation events (Smits *et al*, 2010).

2.1.2.2 Inheritance and evolution

Mammalian mtDNA is inherited maternally (Larsson and Clayton, 1995). The sperm also donate some mtDNA to the fertilized ovum, but it is directed for degradation before further development (Schapira, 2006). There are many thousands of copies of the mitochondrial genome in each cell, a state of which is called polyplasmy (Smeitink *et al*, 2001). Usually, all the mitochondrial genomes are identical in sequence (homoplasmy), but when somatic mutations start to accumulate, e.g. with aging, wild-type and mutant mtDNA molecules start to coexist (heteroplasmy) (Schapira, 2006). Upon cell division, the heteroplasmic mtDNA mutations segregate randomly into daughter cells, which can lead to one tissue or one cell within a tissue having a higher proportion of mutated mtDNA. If the mutation is pathological, it usually requires a minimum threshold level of mutated mtDNAs before a phenotype can manifest (see section 2.4.1). The minimum threshold level of mutant mtDNA depends on the mutation and the tissue being affected (Falkenberg *et al*, 2007). In animals the mutation rate in the mitochondrial genome over evolutionary time is at least 10 times higher than in the nuclear genome (Brown *et al*, 1979). This high mutation rate has been suspected to be due to insufficient means of repairing damaged DNA and the close physical proximity to damaging reactive oxygen species (ROS) inside the mitochondrial matrix (Shadel and Clayton, 1997), although this is debated. Humans have accumulated neutral mitochondrial mutations after the emergence of our species and they have manifest as a series of different DNA sequence polymorphisms. A specific set of polymorphisms in a population is called a mitochondrial haplotype. Using these haplotypes, it has been possible to trace back maternal lineages all the way to a common maternal ancestor, and determine the migration routes used when humans first left Africa (Wallace *et al*, 1999).

2.1.3 The respiratory chain and oxidative phosphorylation (OXPHOS)

Mitochondria are responsible for a number of metabolic functions, the most significant being the production of chemical energy for the cell, in the form of ATP. The end products of glycolysis and fatty acid breakdown are further oxidised in the TCA cycle, which produces reduced electron carrier molecules (NADH and $FADH_2$). These molecules then release their electrons to the respiratory chain complexes, returning to their oxidised state and back to the TCA cycle, enabling further fuel molecule oxidisation. The electrons are passed from one respiratory chain complex to the next, providing the energy that is later used for oxidative phosphorylation (Nelson and Cox, 2008).

The respiratory chain encompasses multisubunit protein complexes I-IV, embedded in the inner mitochondrial membrane, complex V, which is the ATP synthase, and two electron carrier molecules: ubiquinone (coenzyme Q) inside the IMM and cytochrome c in the intermembrane space (Nelson and Cox, 2008). Complex I (NADH dehydrogenase) and complex II (succinate dehydrogenase) transfer their electrons to ubiquinone, which is reduced to ubiquinol. Ubiquinol transfers electrons on to complex III (cytochrome c reductase) and returns to its oxidised state. Complex III then transfers electrons in the intermembrane space to cytohrome c, which then diffuses to interact with complex IV (cytochrome c oxidase, COX), which finally transfers electrons from cytochrome c to molecular oxygen (Boekema and Braun, 2007). During electron transfer, complexes I, III and IV pump protons from the mitochondrial matrix to the intermembrane space. This proton pumping action stores the energy released from the electrons as an electrochemical gradient over the inner mitochondrial membrane. This results in the intermembrane space having a higher proton concentration than the matrix. The IMM thus has an electrochemical potential, utilizing opposing electrical charges (positive outside, negative inside) (Nelson and Cox, 2008). This electrochemical gradient is used as an energy source of complex V (ATP synthase) to produce ATP from ADP and inorganic phosphate. The ATP synthase is of the F_1F_o type (Smeitink *et al*, 2001). The protons flow through the c subunits of the membrane bound section (F_o) of ATP synthase, making it rotate. The rotary movement of the c subunits is transferred to the γ subunit of the section protruding the matrix side (F_1). The γ subunit is a shaft, which is in contact with the catalytic β subunits. There are three β subunits, each either binding strongly to ATP, ADP + P_i or nothing, depending on the orientation of the γ subunit (Nelson and Cox, 2008). This way the proton-motive force generates most of the ATP needed for various purposes (Alberts *et al*, 2008). Finally, ATP needs to be transported outside of mitochondria to places in the cell where it will be

utilised. To accomplish this task, the inner mitochondrial membrane is equipped with an adenine nucleotide translocator that transfers ATP from matrix to cytosol in exchange for ADP (Smeitink *et al*, 2001). An illustration of the respiratory chain structure and function is presented in Figure 2.1.

The respiratory chain was first considered to exist in a fluid state, where all the complexes would diffuse freely in the IMM and oxidative phosphorylation would occur only on random collisions (Nelson and Cox, 2008). Later it was shown first in bacteria and then also in yeast and mammals that the respiratory complexes are associated in various stoichiometric supercomplexes, or "respirasomes" (Schagger and Pfeiffer, 2000). These findings support a solid-state model, in which the respiratory chain complexes are organised so that substrates are channelled straight from one complex to another. Different types of supercomplexes are believed to be present in mammalian mitochondria, most likely of type I_1III_2 and $I_1III_2IV_1$ (Schafer *et al*, 2006; Acín-Pérez *et al*, 2008). The larger supercomplex is more abundant and also has higher activity and stability than the smaller one (Vonck and Schaefer, 2009). The amount of complex IV associated with the respirasome is still unclear: supercomplexes with one, two, four and up to six units of complex IV have been detected (Schagger and Pfeiffer, 2000; Acín-Pérez *et al*, 2008).

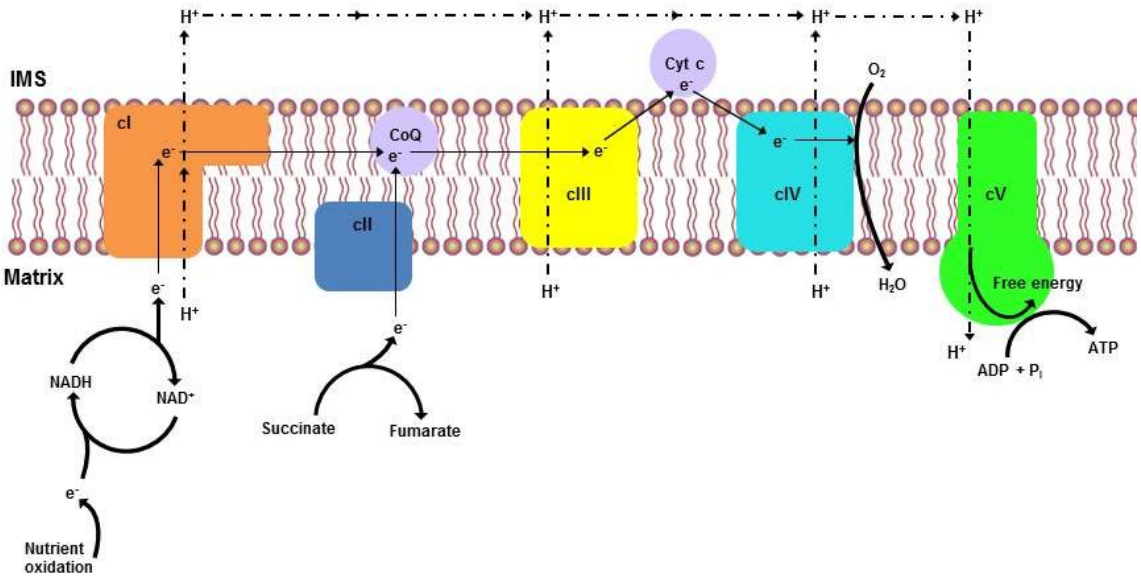

Figure 2.1. The mitochondrial oxidative phosphorylation (OXPHOS) system. Electrons from carbohydrate and other nutrient oxidation reactions are transferred from NADH and FADH (succinate→fumarate reaction) through complex I (cI, NADH dehydrogenase), complex II (cII, succinate dehydrogenase), small electron carrier molecule ubiquinone (CoQ), complex III (cIII, cytochrome c reductase), cytochrome c (Cyt c) and complex IV (cIV, cytohrome c oxidase) to molecular oxygen, which is reduced to water. Concomitantly with the electron transfer, complexes I, III and IV pump protons from the matrix side to the intermembrane space (IMS) and the consequent proton gradient is utilized by complex V (cV, ATP synthase) to form ATP from ADP and inorganic phosphate. Diagram modified from Smeitink *et al*, 2001.

2.2 Cytochrome c oxidase

2.2.1 Structure and biogenesis

Cytochrome c oxidase is the final complex of the mitochondrial respiratory chain. It is a large protein complex, composed of 13 subunits (in mammals), some of which are encoded in each of the two cellular genomes, nuclear and mitochondrial (Khalimonchuk and Rödel, 2005). The nomenclature of COX subunits depends on the organism and in this section the mammalian nomenclature is used, because most of the studies presented here have been carried out on the mammalian enzyme (Fontanesi *et al*, 2008). The three dimensional structure of the mammalian enzyme was solved by Tsukihara *et al* (1996), using X-ray crystallography. The catalytically active core subunits (Cox1, Cox2 and Cox3) are encoded by the mitochondrial genome. The bacterial enzyme is composed of only these three subunits and they share sequence homology with the mitochondrially encoded subunits. The 10 other subunits of the mammalian COX are encoded in the nuclear genome, translated in the cytosol and imported into mitochondria for assembly into complex IV (Stiburek *et al*, 2006).

2.2.1.1 Mitochondrially encoded subunits

Cox1 is the largest of all COX subunits and it is anchored tightly into the IMM with 12 transmembrane helices. Cox1 also coordinates the electron transfer centres and proton pumping channels (D- and K-channels) (see section 2.2.2) (Stiburek *et al*, 2006). The electron transfer centres are unique for COX because they fuse a heme group and copper atom. In Cox1 there are two catalytic centres: heme *a* and binuclear heme a_3-Cu_B centre (Fontanesi *et al*, 2008). Cox2 is the smallest of the core subunits and it is also anchored to the IMM via transmembrane α-helical hairpin. The C-terminal head of Cox2, composed of a 10 stranded β-barrel, projects into the intermembrane space and contains the Cu_A-centre and cytochrome c docking site. The last core subunit, Cox3, is very hydrophobic and it is anchored to the IMM with 7 transmembrane helices (Stiburek *et al*, 2006). Cox3 does not have prosthetic groups, but it seems to accomplish the rapid proton flow through the D-channel of Cox1 (Hosler, 2004).

2.2.1.2 Nuclear-coded subunits

The remaining ten nuclear-coded mammalian subunits of COX are required for synthesis and stability of the enzyme. They are also thought to regulate the activity of the COX complex. Most of the nuclear subunits are also transmembrane proteins, except for subunits Cox5a and Cox5b, which are located on the matrix side, and subunit Cox6b, which associates with the complex in the

intermembrane space. Some of the nuclear subunits have tissue-specific isoforms (usually heart/muscle or liver specific isoforms), including Cox4, Cox6a, Cox6b, Cox7a and Cox8 (Fornuskova *et al*, 2010). Subunit 5b is known to be in contact with both Cox1 and Cox2 from the matrix side. The subunit includes a zinc binding site, but the significance of this motif is not known. It has also been noticed that the subunit 5b content of different tissues can vary, even within different compartments of one specific tissue. Usually tissues with high energy demand have higher Cox5b content (Galati *et al*, 2009). COX subunit 4 is the largest of the nuclear encoded subunits, and it has tissue-specific isoforms encoded by separate genes (Fornuskova *et al*, 2010). Cox4 mediates allosteric inhibition of the complex by ATP, which binds to the matrix portion of the subunit (Arnold and Kadenbach, 1997).

2.2.1.3 Biogenesis of cytochrome c oxidase

Before the assembly of COX can start, the accessory groups have to be inserted into core subunits and nuclear subunits have to be imported to the site of enzyme formation (Stiburek *et al*, 2006). Not all of the protein factors or intermediate steps in COX biogenesis are currently known, but there has been a lot of research on the subject in recent years. Although more auxiliary proteins are being identified, the specific functions of only a few are known (Soto *et al*, 2012). There are a number of factors required for the formation of heme and copper centres in Cox1 and Cox2. The heme *a* rings are synthesised sequentially from heme *b* rings by a series of enzymes, including Cox10 and Cox15 (Khalimonchuk and Rödel, 2005). They are likely inserted into the Cox1 subunit early in COX biogenesis before addition of other subunits, and the insertion seems to be assisted by the COX biogenesis regulator protein Surf1 (Ylikallio and Suomalainen, 2012). Cox17 and Cox19 are small hydrophilic proteins that function upstream in the pathway that inserts Cu-atoms to COX. The downstream copper-binding proteins associated with copper delivery to Cu_A centre of Cox2 are Sco1 and Sco2. The corresponding copper binding protein delivering copper to Cu_B of Cox1 is Cox11 (Stiburek *et al*, 2006).

In the initial stages of COX assembly, Cox1 becomes associated with proteins that are not present in the mature COX, constituting the first subcomplex intermediate (S1). After incorporating Cox1 into IMM, Cox1 associates with Cox4/Cox5a heterodimer, forming the subcomplex intermediate S2. Cox2 then joins this subcomplex. Cox2 association is a prerequisite for Cox3 association (Stiburek *et al*, 2006), which is accompanied by the incorporation of many nuclear subunits, including Cox5b, Cox6c, Cox7b, Cox7c and Cox8 (intermediate S3). A recent study by Fornuskova *et al* (2010) suggested that in the late stage of COX assembly, subunits Cox7a and Cox6b are added

to intermediate S3 prior to the last subunit Cox6a, in two separate steps (producing the complete 13-subunit enzyme, denoted as S4). The final, mature form of COX is achieved after some modifications (Cu_B centre coordinating amino acids are linked) and respiratory supercomplex formation (Khalimonchuk and Rödel. 2005). The structure of the respiratory supercomplexes was discussed in section 2.1.3. The COX biogenesis pathway is depicted in Figure 2.2.

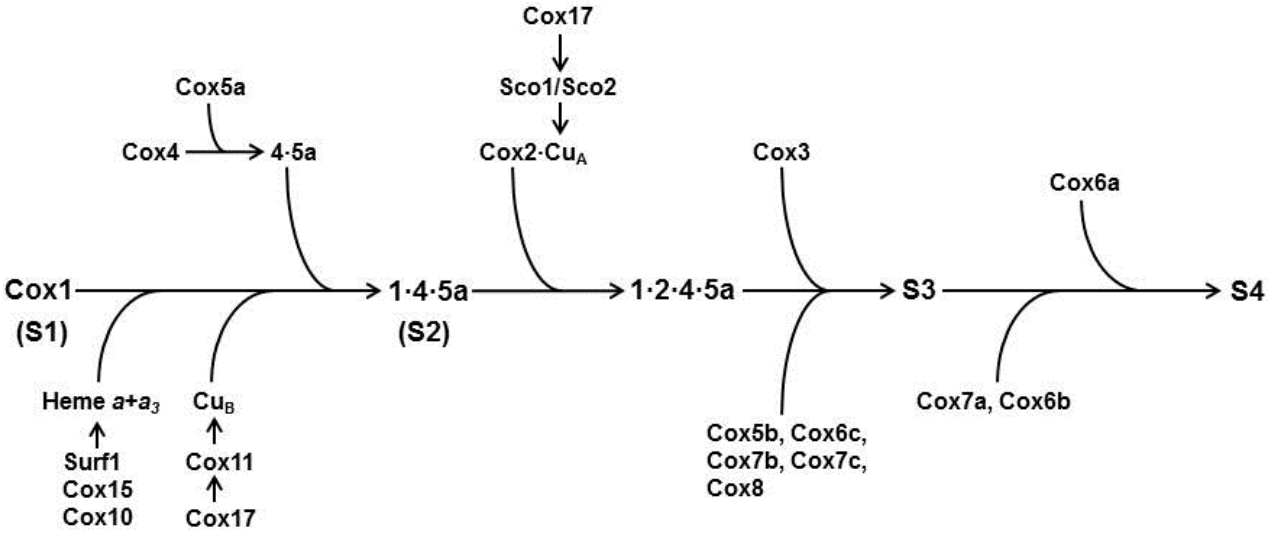

Figure 2.2. COX assembly pathway. In addition to all the subunits, prosthetic groups and enzymes/proteins needed for their preparation are presented in the picture, in the propoced sequential order. S1, S2 and S3 denote the subcomplex intermediates and S4 represents the final 13-subunit enzyme complex. Picture modified from Fornuskova *et al*, 2010.

2.2.2 Modes of action: Transmission of electrons and proton translocation

The electron transfer reactions and proton translocation have been studied in great detail. This has revealed that the reduction of oxygen to water actually happens in two phases: the reductive and oxidative phase (Yoshikawa *et al*, 2011; Yoshikawa *et al*, 2012). The reductive phase is composed of the electron transfer reactions from the metallic centres to molecular oxygen. The oxidative phase consists of the subsequent water formation and proton translocation (Stiburek *et al*, 2006).

In the reductive phase, electrons are transferred to the heme a_3-Cu_B centre, which needs to be fully reduced in order for molecular oxygen to bind to it. Electrons released from cytochrome c to Cu_A centre are quickly distributed to heme *a* and from there to the heme a_3-Cu_B centre (Stiburek *et al*, 2006). The reduced heme a_3-Cu_B centre binds O_2, which is guided to Cu_B by an O_2 transfer pathway in Cox3. O_2 receives one electron from Fe^{2+} of heme a_3 and forms O_2^-, which stays bound to Fe_{a3}^{2+}. The rest of the electrons are transferred to O_2^- after a water molecule has been fixed to the reaction site in order to produce two H_2O molecules out of O_2 (Yoshikawa *et al*, 2012).

The side of the enzyme facing the matrix has hydrophilic pores that are thought to function in proton translocation. Proton translocation through the channels happens with the aid of hydrogen bonds that form between water molecules and hydrophilic amino acids lining the channel (Tsukihara *et al*, 1996). Two of these cavities presumably form pathways connecting to the O_2 reduction site. These pathways are named the D- and K-pathways and they supply the protons used to generate water from O_2 (Yoshikawa *et al*, 2011). The D-pathway is probably branched, because it can direct protons to the active site of the enzyme and to the other side of IMM (Tsukihara *et al*, 1996; Yoshikawa *et al*, 2011). There is also a third pathway, the H-pathway. However, the H-pathway is thought to function only in transferring protons to the IMS (Yoshikawa *et al*, 2011). All of the pathways reside inside Cox1, which has many transmembrane helices that form hydrophilic pores (Tsukihara *et al*, 1996).

2.3 Alternative respiratory enzymes

2.3.1 Alternative oxidase

A distinctive feature between the respiratory chains of mammals and plants is that the plant respiratory chain can be branched, i.e. there are more potential routes for electrons than the one depicted above in section 2.1.3. Branching of the respiratory chain is enabled by alternative respiratory enzymes, the most relevant being the cyanide-resistant alternative oxidase (AOX). AOX is a single subunit protein that transfers electrons from ubiquinol to molecular oxygen reducing it to H_2O, without pumping protons from the matrix to the intermembrane space (Rustin and Jacobs, 2009). Thus, it bypasses complexes III and IV of the respiratory chain, and the ATP production capacity of mitochondria is therefore lowered (McDonald *et al*, 2009). However, AOX has the ability to keep the respiratory chain (and proton pumping through complex I) active in situations where the cytochrome pathway is blocked. AOX is thought to exist as a homodimer, although monomers are known to exist and function in physiological conditions (Siedow and Umbach, 2000). Most structural studies performed on AOX have involved strategies utilizing mutagenesis of the polypeptide chain (Albury *et al*, 2002), and there has been no crystal structure data of the enzyme until very recently (Shiba *et al*, 2013). The interlocked cylindrical monomers present a hydrophobic face on one side and a hydrophilic face on the other. Association into the IMM most likely happens in an interfacial fashion via the hydrophobic surface. The active site of the enzyme is a non-heme di-iron centre, buried within a four-helix bundle. Four glutamate and two histidine residues coordinate the iron atoms (Shiba *et al*, 2013). AOX stays inactive in normal physiological conditions where cytohrome pathway is not blocked, because the high Km value of AOX for

reduced quinones does not allow it to compete with the cytochrome pathway (Rustin and Jacobs, 2009). Activity of the enzyme is regulated by metabolic intermediates, particularly α-keto acids (e.g. pyruvate) and succinate (Siedow and Umbach, 2000).

The alternative oxidase is widely found in plants and fungi, but also in some protists and also recently in animals (McDonald *et al*, 2009). However, AOX seems to be present only in relatively primitive animal phyla, as it is not found in vertebrates or arthropods (Rustin and Jacobs, 2009). AOX is thought to provide metabolic flexibility to plants that live in variable developmental and environmental conditions (Berthold *et al*, 2000). AOX expression has also been shown to lower ROS production in plants (Maxwell *et al*, 1999). In animals, AOX expression has been proposed to allow worms to inhabit environments with low pH and toxic sulphites (McDonald *et al*, 2009). AOX may also suppress ROS production by preventing over-reduction of the respiratory chain in situations where COX is inhibited (e.g. plant parasites that encounter cyanogenic compounds excreted by the host plant, that inhibits COX) (McDonald *et al*, 2009).

It has been proposed that AOX could be used to alleviate symptoms of mitochondrial diseases, because it naturally functions in highly reducing conditions that are also observed in patients with mitochondrial diseases. Therefore there has been considerable effort to test whether AOX could be expressed beneficially in mammalian cells and in mitochondrial disease model animals, mainly in *Drosophila melanogaster* and mouse (Rustin and Jacobs, 2009). The AOX gene from the ascidian *Ciona intestinalis* has been expressed in human cells and it has been shown to confer cyanide resistance as well as compensate the deleterious effects of cytochrome c oxidase deficiency (Hakkaart *et al*, 2006; Dassa *et al*, 2009b). More recently, a mouse model expressing AOX was developed (El-Khoury *et al*, 2013). In the paper, AOX expression was shown to confer cyanide resistance and decrease ROS production in mice with respiratory chain blockage. The *Drosophila melanogaster* models expressing AOX are discussed in section 2.3.3. The tests done on the human cell and whole animal models indicate that AOX is a useful tool for compensating symptoms resulting from respiratory chain deficiencies and therefore it should be considered (long-term) as a gene therapy tool for mitochondrial diseases in future.

2.3.2 Alternative NADH dehydrogenase

The mammalian complex I, which is composed of 45 different subunits (Lazarou *et al*, 2009), is replaced in the budding yeast, *Saccharomyces cerevisiae*, with a single polypeptide from the NDI family. Ndi1 is a single subunit rotenone-insensitive NADH-quinone oxidoreductase, which

associates into the matrix side of the IMM. Ndi1 does not couple electron transfer to proton pumping (Yagi *et al*, 2001). It is the primary entry point of electrons of the yeast respiratory chain. The same type of enzyme (bypassing complex I but not translocating protons) is found in plants and bacteria, but not in mammals (Iwata *et al*, 2012). Ndi1 is also an alternative respiratory enzyme, because it can bypass complex I of the mammalian respiratory chain. It has been proposed that Ndi1 could also be utilized as a therapeutic tool to compensate complex I deficiency in mitochondrial disease patients (Yagi *et al*, 2001).

The yeast Ndi1 exists as a homodimer under physiological conditions, much like AOX. On one side of the dimer there is a hydrophobic ridge that is lined with positively charged patches. This is most likely the surface that anchors the enzyme into the IMM (Iwata *et al*, 2012). The active site of the enzyme features an FAD cofactor that is bound to the enzyme noncovalently. Unlike the mammalian complex I, Ndi1 does not have any iron-sulfur clusters (Yagi *et al*, 2001). The binding sites for NAD^+ and quinone are overlapping, which suggests that the electron transfer reactions happen in a ping-pong mechanism (Iwata *et al*, 2012). Ndi1 seems also to operate according to a two-electron reaction mechanism, whereas complex I works via a one-electron mechanism (Yagi *et al*, 2001).

As stated above, Ndi1 has also been studied as a potential therapeutic tool for complex I deficiency. In fact Ndi1 has been expressed in human cells and it has supported the growth and survival of cells even in conditions where complex I was inhibited (Seo *et al*, 2000). Ndi1 has also been introduced in the optic lobe of central nervous system in rats by means of gene transfer (Marella *et al*, 2010). In the experiment, vision of rats suffering from Leber's hereditary optic neuropathy (which is due to mutations in genes coding for complex I subunits) was restored to normal level after introduction of Ndi1, even though it was severely impaired because of the disease. This experiment was a direct example of the possibilites of gene therapy utilizing alternative respiratory enzymes against respiratory chain dysfunctotion. Studies concerning fruitfly models of mitochondrial disease with Ndi1 expression are discussed below.

2.3.3 Alternative respiratory enzymes in *D. melanogaster*

Drosophila models expressing alternative oxidase both constitutively and inducibly has been generated in Howard Jacobs' laboratory (Fernandez-Ayala *et al*, 2009). The enzyme was shown to target mitochondria but to remain enzymatically inactive under normal physiological conditions. AOX expression had minimal effect on the normal phenotype of flies, with only a slight weight loss

in young adults. It was able to alleviate the symptoms of respiratory chain diseases, modeled by *Surf1* knockdown (which results in COX deficiency) and knockdown of the *Drosophila* homolog of the Parkinson's disease gene *dj-1*β. A few studies have been made where AOX has been used to rescue OXPHOS dysfunction specifically in the nervous system of flies (Humphrey *et al*, 2012). However, in this case OXPHOS deficiency was achieved by downregulating the catalytic subunit of mtDNA polymerase.

The yeast alternative NADH dehydrogenase Ndi1 has also been introduced into the *Drosophila* genome and the expression of the transgene was shown to be harmless for the fly (Sanz *et al*, 2010b). Ndi1 expression was also able to rescue the detrimental phenotypes resulting from complex I knockdown (Cho *et al*, 2012; Sanz *et al*, 2010b). Neuronal expression of Ndi1 was also reported to extend the lifespan of flies with no OXPHOS dysfunction (Bahadorani *et al*, 2010).

Ndi1 expression should not have any beneficial effect on flies with COX knockdown because Ndi1 cannot compensate for the oxygen-reducing function of COX. Therefore in this study Ndi1 was used, in principle, as a control for flies coexpressing AOX under conditions of COX knockdown.

2.4 Mitochondrial diseases

Mitochondrial diseases commonly refer to disorders of the respiratory chain, which by estimate have a prevalence of 1 in 5 000 people (Ylikallio and Suomalainen, 2012; DiMauro, 2004). Mitochondrial diseases are a diverse group of disorders with a variety of symptoms. The disorders can cause symptoms in any tissue at any stage, but usually they affect organs with high energy demand, most commonly muscle and nervous system (DiMauro and Schon, 2008; Ylikallio and Suomalainen, 2012). To date there is no cure for mitochondrial diseases, only supportive measures that alleviate symptoms are clinically in use (Schapira, 2006).

2.4.1 Molecular pathology

Because of the dual genetic origin of the respiratory chain complexes, mitochondrial diseases can arise from mutations in OXPHOS complex genes encoded in either the mitochondrial or nuclear genome (DiMauro, 2004). Mutations in genes that code for proteins that participate in other mitochondrial functions can also cause mitochondrial diseases. These functions include mitochondrial protein synthesis, synthesis of assembly factors for OXPHOS complexes, import of cytoplasmically produced proteins, intergenomic signalling, and mitochondrial dynamics, such as fusion, fission and distribution (DiMauro, 2004; DiMauro and Schon, 2008). The major molecular

event behind the symptoms of mitochondrial diseases is believed to be ATP deficiency. When one or many of the respiratory chain complexes have a defect, ATP production is decreased, which can lead to many secondary problems. Another major cause of the symptoms is lactic acidosis, which is due to the secondary blockage of pyruvate dehydrogenase enzyme function. If the enzyme function is blocked, excess pyruvate is converted to lactate and transported out of the cells. This will lead to drop in pH in tissues of respiratory chain deficiency and to other secondary problems (Ylikallio and Suomalainen, 2012).

As stated above, mtDNA exist in hundreds or thousands of copies in single cells. In a homoplasmic state, all the mtDNA molecules are identical, but in the heteroplasmic state mutant and wild-type mtDNA molecules coexist (Ylikallio and Suomalainen, 2012). In order for a mitochondrial disease to manifest, a certain proportion of the mtDNA molecules have to be mutated (DiMauro, 2004). This is called the threshold effect. The threshold for different diseases and even for different tissues varies. This is because a mutation in one tissue can lead to manifestation of a disease whereas the same mutation in another tissue can be harmless (Leonard and Schapira, 2000a). In some instances, the level of mutated mtDNA can affect the symptoms and nature of the disease. For example, in the case of mutation T8893G, if the degree of heteroplasmy is moderate (about 70 %) the mutation inflicts a subacute or chronic disease of young adults called NARP (neurogenic weakness, ataxia, retinitis pigmentosa) but if the level of heteroplasmy is very high (around 90 %), it causes Leigh syndrome, a rapidly progressive encephalopathy of infancy or early childhood (DiMauro, 2004).

Mitochondrial diseases are often categorized according to where the mutated gene lies (i.e. either mitochondrial or nuclear genome) (Leonard and Schapira, 2000a, 2000b; Ylikallio and Suomalainen, 2012). The most common disease caused by mutation in mtDNA is Leber hereditary optic neuropathy (LHON) (Schapira, 2006). It is primarily a disease of young men, which is characterized clinically by loss of vision due to degeneration of the retinal ganglion cells and their axons. The mutated genes in this disease code for core subunits of complex I and they are usually present in homoplasmic or high mutant heteroplasmic proportions. Other common mitochondrial diseases caused by mutations in mtDNA are MERRF and MELAS. Both diseases are encephalomyopathies, diseases that affect both nerve and muscle. MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) is more common of these two. The disease is usually caused by a common mutation (A3243G) in the gene $tRNA^{Leu}$, although many other mutations can also cause the disease (DiMauro, 2004). MELAS presents usually in children and adolescents and the symptoms include vomiting, seizures and dementia (Leonard and Schapira,

2000a). MERRF stands for myoclonus epilepsy with ragged red fibers, and typically affects young adults. Three mutations, all in the gene $tRNA^{Lys}$, have been associated with the disease (DiMauro, 2004). The disease is characterized by seizures, mitochondrial myopathy, and cerebellar ataxia. Among the diseases that are usually due to mutations in the nuclear genome, is COX-deficient Leigh's syndrome, which is due to mutations in genes coding proteins required for COX assembly and biogenesis (discussed in more detail below) (Ylikallio and Suomalainen, 2012).

Mitochondrial dysfunction has been identified in a number of neurodegenerative diseases including Parkinson's disease, Alzheimer disease and Huntington's disease (Schapira, 2006; Federico *et al*, 2012), although the mechanism by which it contributes to pathology are under debate. The accumulating mutations in mtDNA of aging neurons are thought to produce the respiratory chain dysfunction and the sequential ROS production that leads to neuron degeneration. Several mitochondrially targeted proteins seem to be involved in Parkinson's disease pathology when mutated, including DJ-1 and PINK1. DJ-1 potentially acts as an antioxidant. Mutations in *DJ-1* cause increase in ROS production (Federico *et al*, 2012). In Alzheimer disease, oxidative stress may activate signalling pathways that alter amyloid precursor protein processing, which ultimately leads to plaque formation (a characteristic symptom of Alzheimer disease) (Lin and Beal, 2006).

2.4.2 Cytochrome c oxidase related mitochondrial diseases

COX deficiency is the most common cause of pathological respiratory chain defects. The diseases most often affect infantile or young children (Robinson, 2000). Mutations of the mtDNA-encoded core subunits of COX are rare, but there have been reports of *COX1-3* mutations causing myopathy and MELAS in patients (Diaz, 2010; Ylikallio and Suomalainen, 2012). So far, only one incident of COX dysfunction caused by a mutation in a nuclear-coded subunit has been reported (Massa *et al*, 2008). In this report, two siblings were diagnosed with severe infantile encephalopathy, which was due to a homozygous mutation in the *COX6b1* gene. However, majority of the mutations causing COX deficiency affect the genes coding for auxiliary proteins. For example, mutations in assembly factor Surf1 are associated with COX-deficient Leigh syndrome. The function of Surf1 is not fully known yet, but it is thought to be involved in inserting heme *a* into Cox1 (Ylikallio and Suomalainen, 2012). The pathology of Leigh syndrome includes necrotic lesions in subcortical areas of the brain that lead into e.g. ophthalmoparesis, nystagmus, ataxia and dystonia (Diaz, 2010). COX activity in the affected tissues reduces to 10-20 % of the normal level (Robinson, 2000). Another example of mutated auxiliary proteins leading to COX deficiency is Sco1 and Sco2 mutations. Sco2 mutation inflicts fatal infantile COX deficiency with the major symptoms being

encephalopathy and hypertrophic cardiomyopathy (Stiburek *et al*, 2005). On the other hand, Sco1 mutations seem to cause lactic acidosis and hepatic failure (Diaz, 2010). Altogether, COX deficiencies can cause a variety of symptoms, of which Leigh syndrome or Leigh-like diseases, cardiomyopathy, and liver failure are most common (Ylikallio and Suomalainen, 2012).

2.5 *Drosophila melanogaster* animal model

Drosophila melanogaster has had a major influence in biological studies, most importantly in the fields of genetics and developmental biology (Sánchez-Martínez *et al*, 2006). Many features of the *Drosophila* genome and biology still make it a good model to study many human diseases including neurodegenerative and mitochondrial diseases (Bilen and Bonini, 2005; Jacobs *et al*, 2004).

2.5.1 Development and genomics of *D. melanogaster*

The common fruit fly, *Drosophila melanogaster*, possesses a short generation time. A new fly develops from egg to adult in ten days in 25°C (Klug *et al*, 2009). However, development is accelerated at higher temperatures and slowed when growth temperature is decreased. Before adulthood the fly has to go through several stages of development, which are the embryonic, three larval stages (1^{st}-3^{rd} instar) and pupal stage. Each larval stage lasts for 24 hours and metamorphosis inside the puparium takes 4 days to complete. The adult fly is approximately 2-3 mm long, females are slightly larger than males (Ashburner *et al*, 2005). Females and males also differ in their colouration: males have a dark end of the abdomen, whereas females have a striped abdomen. Male flies have a row of black hairs in the middle of the first legs called sex combs that are missing in females, and which are used during copulation to attach the female. Furthermore, males have visible black hairs surrounding the reproductive parts beneath the abdomen.

The full 180 Mb *Drosophila* genome has been sequenced (Adams *et al*, 2000). The genome is organized into three autosomes and one sex chromosome. One of the autosomes is considerably smaller than the other two (only 1 Mb in size). The genome codes for roughly 13 600 proteins. *Drosophila* has all the major cell and tissue-types as humans, including a central nervous system composed of brain and axonal nerve trunk (central nerve cord). The mitochondrial genome in flies codes for the same proteins and RNA molecules as it does in vertebrates (Koc *et al*, 2001a, 2001b). All of the components of the respiratory chain, both structural and auxiliary proteins, as well as the apparatus for mitochondrial genome replication, genome maintenance, and mitochondrial protein synthesis and transport are conserved from insects to humans.

2.5.2 *D. melanogaster* as a model for human diseases

There are several factors that make *Drosophila* useful as a model to study human diseases. First of all it is small and large stocks and experiments are relatively easy and cheap to maintain. The whole genome of the fly is known and today we possess many genetic tools and techniques that allow specific manipulations, including gene knockdowns and knock-ins. Non-recombining balancer chromosomes further aid in these manipulations. Lastly, phenotypic characters can be quantified by carefully designed experiments (Jacobs *et al*, 2004; Sánchez-Martínez *et al*, 2006). Because of the long and continuous use of *Drosophila*, many biological pathways have been elucidated in the fly and it has spurred the creation of many publicly available databases (FlyBase, FlyAtlas).

Drosophila has been used to study neurodegenerative diseases, cancer, cardiac pathologies and mitochondrial diseases (Sánchez-Martínez *et al*, 2006). Humans and flies share many fundamental biological pathways, although some are simplified in flies (Jacobs *et al*, 2004). As stated above, both have similar cell types that compose similar organ and organ systems. *Drosophila* has also been shown to possess orthologs for ~60 % of genes that are known to cause diseases in humans (Rubin *et al*, 2000).

The neurodegenerative disease models include those for Parkinson's disease, Alzheimer disease and Huntington's disease (Bilen and Bonini, 2005). Parkinson's disease has been modelled by mutating *parkin* gene in flies, which results in dramatic degeneration of muscle cells with deformed mitochondria. The flies also present an abnormal wing phenotype, but no loss of dopaminergic neurons. Another Parkinson's disease model was made by mutating the gene *dj-1β*, a fly homolog of *DJ-1*, a gene that is associated with Parkinson's disease when mutated (Fernandez-Ayala *et al*, 2009). Alzheimer disease has been modelled by expressing the mutant human amyloid precursor protein in flies (Bilen and Bonini, 2005). The same type of strategy was used in a study by Bodai *et al* (2012), when mutant huntingtin protein was expressed in flies.

Mitochondrial diseases have been simulated extensively on *Drosophila*. One such model is the *technical knockout* mutant fly (*tko*), which has a mutation in the mitochondrial gene coding for mitoribosomal subunit S12 (Toivonen *et al*, 2001). The mutation causes impaired mitochondrial protein synthesis, leading to a phenotype characterized by developmental delay, bang sensitivity (paralysis through mechanical stress) and deafness. Studying *tko* flies can produce answers as to what happens in mitochondria with insufficient protein synthesis (Jacobs *et al*, 2004). Many studies

have taken advantage of available methods to manipulate the *Drosophila* genome and produce mitochondria specific gene knockdowns (Zordan *et al*, 2006; Fernandez-Ayala *et al*, 2009). These methods inlcude post-transcriptional gene silencing via RNA interference (RNAi) and expression of RNAi constructs with binary GAL4 - upstream activating sequence (UAS) system. Both of these methods were used in this study and they are further described below.

2.5.2.1 Transgenic RNAi fly lines

RNA interference (RNAi) is best known as a way for cells to silence unwanted gene expression via small complementary RNA molecules. The mechanism is also used by cells to protect against transposable DNA elements and also to regulate chromatin structure (Moazed, 2009). There are actually different types of RNAi events, depending on the RNA molecule that is used as a template to direct gene silencing. All of the "classic" small RNA molecules are 20-30 nucleotides long (Krebs *et al*, 2011). They are produced from longer double-stranded precursor molecules by a ribonuclease called Dicer. Small RNA molecules are then loaded into the RNA-induced silencing complex (RISC), which is the protein responsible for target RNA molecule degradation. The RISC complex is built around the Argonaute core protein (Krebs *et al*, 2011). Argonaute binds the small guiding RNA and it also possesses an RNase H type nuclease domain that cuts the target molecule (Moazed, 2009).

Vienna *Drosophila* RNAi center (VDRC) holds a library of transgenic RNAi fly lines that are publicly available. The library encompasses over 30 000 different transgenic lines that together cover almost 94 % of fly genes. The transgenic flies express a double-stranded hairpin RNA (shRNA) molecule that guides the RISC complex to a specific target RNA (Dietzl *et al*, 2007). The shRNA sequence is complementary to the gene sequence that is targeted for knockdown. The transgenic UAS-RNAi fly lines were first created using P-element-based method, which utilized random insertion of the transgene into the fly genome. The problem with this method is the high occurrence of false negatives due to insertion of the transgene in the middle of another vital housekeeping gene. To eliminate the false negative, another method based on phiC31 site-specific integration was developed (Ni *et al*, 2007). This method allows insertion of the UAS-RNAi transgene to specific site in the fly genome. Both P-element and phiC31 derived transgenic lines are represented in VDRC. In both cases, expression of the UAS-RNAi transgene needs to be induced by the binary GAL4-UAS system.

2.5.2.2 GAL4-UAS expression system

The GAL4-UAS system was originally found from budding yeast *Saccharomyces cerevisiae*, where its function is to induce gene expression in situations when galactose is the only available source of catabolic energy (Krebs *et al*, 2011). *GAL* genes code for a group of enzymes related to galactose metabolism. All the *GAL* genes are under the control of the UAS enhancer, which itself is under the control of a positive regulator, GAL4. GAL4 has a DNA-binding zinc-finger domain, and binds to DNA as a dimer. The GAL4-UAS system has later on been adapted for use to drive the heterologous expression of transgenes both in flies and also in human cells (Brand and Perrimon, 1993; Webster *et al*, 1988). Both the UAS site and GAL4 have been mutagenized in order to establish an optimal binding site for GAL4 and also to achieve efficient transcription activation in flies (Brand and Perrimon, 1993). Tissue specific or constitutively expressed GAL4 and the UAS-transgene are carried in different parental fly lines and the transgene expression is induced in the progeny flies after crossing (McGuire *et al*, 2004). This allows the viability of parental lines carrying toxic products since neither expresses the transgene by itself i.e. UAS-transgene lines do not have the expression enhancer and the driver lines do not carry any transgene to drive (although they express GAL4 in specific tissues or developmental stages). Other advantages include the versatility of the system: any GAL4 driver line can be crossed with any UAS transgene line to induce expression. There are thousands of available GAL4 lines and they have been pooled in the Gal4 Enhancer Trap Insertion Database (GETDB) (Hayashi *et al*, 2002). With the enhancer trap system, GAL4 is inserted in the genome randomly under the influence of generic tissue specific enhancers (McGuire *et al*, 2004). The only drawback of this system is that the expression cannot be fully regulated. GAL4 expression is guided by the enhancer and the UAS-transgene is expressed whenever there is GAL4 present.

In order to induce transgene expression at specific time and place, several modifications of the GAL4-UAS system have been developed. One of these modifications takes advantage of the natural repressor of GAL4, GAL80. In yeast when galactose is absent, GAL80 binds to GAL4 and prevents it from binding DNA (Krebs *et al*, 2011). When galactose is present, it releases GAL80 from GAL4 and GAL4 is free to induce expression of *GAL* genes. A temperature-sensitive variant of GAL80 (GAL80ts) has been constructed for expression under the *tub*-promoter in *Drosophila* (McGuire *et al*, 2004). In this system, GAL80ts is ubiquitously expressed and thus disables GAL4. The expression is induced when GAL80ts is degraded by placing flies in high temperature (approx. 32°C). The system is named TARGET for temporal and regional gene expression targeting.

Another system combines GAL4 with a hormonal response. Two chimeric proteins have been constructed: one is a GAL4-estrogen receptor fusion (GAL4-ER) and the other is a GAL4-progesterone receptor fusion (GeneSwitch). In the GeneSwitch system, expression is induced by feeding the flies with an anti-progestin drug, RU486. The benefit of this system is that the expression level can be controlled by varying the concentration of RU486 that is fed to the flies. Other systems include the FLP recombinase recognition-GAL4-UAS system, in which a transcriptional-termination cassette is inserted between the promoter and GAL4 or between UAS and transgene. The cassette is removed by FLP recombinase, which is cloned downstream of a heat-shock promoter. Thus expression can be induced by heat-shock treatment. However, this treatment can be done only once and the transgene expression stays active thereafter (McGuire *et al*, 2004).

3. AIMS OF THE RESEARCH

The aim of this master's thesis is to

1. Characterize the phenotype of nervous system specific knockdown of cytochrome c oxidase in *Drosophila melanogaster* and
2. Test whether alternative oxidase can rescue the deleterious phenotypes.

The hope is that the results will give more insights into the pathology of COX deficiencies and provide a better understanding of whether/how AOX function can alleviate the pathological phenotypes. With the help of this information, AOX can one day be evaluated as a potential therapeutic tool for OXPHOS disorders.

4. MATERIALS AND METHODS

4.1 Fly stocks and maintenance

4.1.1 Fly stocks

Drosophila melanogaster driver lines are presented in Table 4.1 with full genotype and genomic location. Homozygous Nrv2-GAL4 and elav(3)-GAL4 stocks were crossed with $w^{-};\frac{CyO}{2}$ and $w^{-};\frac{Tm3,Sb}{3}$ balancer stocks, respectively, in order to establish balanced driver lines. The UAS-RNAi lines from Vienna *Drosophila* RNAi center (VDRC) are shown in Table 4.2 with genomic location, insertion type and target gene of silencing. In addition, existing stocks of flies expressing both an RNAi construct and a transgene (AOX or Ndi1) were used as well as GFP reporter lines. These stocks are shown in Table 4.3 with their notation, genotype, phenotypic character and a reference to the original paper where AOX or Ndi1 transgenic lines were created.

4.1.2 Fly food media

Fly stocks and all the experiments were carried out in standard high sugar fly food medium, composed of 1.5 % (w/v) sucrose (Sigma-Aldrich), 3 % glucose (AnalaR Normapur®, VWR), 3.5 % active dry yeast, 1.5 % maize flour, 1 % wheat germ, 1 % soya flour, 1 % agar (Oriola) and 3 % treacle, to which was added 0.1 % Nipagin M (Sigma-Aldrich) and 0.5 % (v/v) propionic acid (Acros Organics).

4.1.3 Environmental conditions

During the characterization of knockdown phenotype and producing flies for imaging, flies were kept in incubators at 25°C with a 12 h-12 h day-night cycle. Stock bottles were kept in the fly laboratory at room temperature.

Table 4.1. Neural specific GAL4 driver lines. Line name, genotype, genomic location and comments.

Driver line (BL number)	Genotype	Genomic location	Comments
Nrv2-GAL4 (6800)	*w*; P{nrv2-GAL4.S}3*	2	GAL4 expressed exclusively in the nervous system
elav-GAL4 (8760)	*w*; P{GAL4-elav.L}3*	3	Expresses GAL4 in the nervous system
elav-GAL4 (458)	*P{GawB}elavC155*	X	GAL4 expressed in all tissues of the embryonic nervous system beginning at stage 12

Table 4.2. RNAi construct lines. Transformant ID, genomic location, insertion type and gene targeted for silencing.

Transformant ID	Genomic location	Insertion type	Gene targeted for silencing
109338	X	Targeted	CoIV, COX IV
30892	3	Random	CoVb, COX Vb

Table 4.3. Existing transgenic *D. melanogaster* lines. Line name, genotype, genomic location of transgene(s) and phenotype

Line	Genotype	Genomic location	Comments
COX IV-KD;AOX	$\frac{COX\,IV\text{-}KD}{COX\,IV\text{-}KD};\frac{UAS-AOX^{F24}}{UAS-AOX^{F24}}$	2 (RNAi), 3 (AOX)	Fernandez-Ayala *et al*, 2009
COX IV-KD;Ndi1	$\frac{COX\,IV\text{-}KD}{COX\,IV\text{-}KD};\frac{UAS\text{-}Ndi1^{A40}}{UAS\text{-}Ndi1^{A40}}$	2 (RNAi), 3(Ndi1)	Sanz *et al*, 2010b
COX Vb-KD;AOX	$\frac{UAS\text{-}AOX^{F6}}{UAS\text{-}AOX^{F6}};\frac{COX\,Vb\text{-}KD}{COX\,Vb\text{-}KD}$	2 (AOX), 3 (RNAi)	Fernandez-Ayala *et al*, 2009
COX Vb-KD;Ndi1	$\frac{UAS\text{-}Ndi1^{B10}}{UAS\text{-}Ndi1^{B10}};\frac{COX\,Vb\text{-}KD}{COX\,Vb\text{-}KD}$	2 (Ndi1), 3 (RNAi)	Sanz *et al*, 2010b
stinger-GFP	w^{1118};*UAS-stinger-GFP*;+	2	Viable and fertile
mCD8-GFP	$y^{*}w^{*};\frac{UAS\text{-}mCD8\text{-}GFP}{CyO}$	2	Viable and fertile, curly wings

4.2 Determination of COX knockdown phenotype with and without AOX rescue

4.2.1 Lethality assay

Three replicates of each individual cross were made and 10-12 virgin females and 5-6 males were used in each cross. Parental flies were left to copulate for three days, after which they were transferred to fresh fly food vials and left to copulate for two days. Finally the parental flies were discarded. Vials were kept in the +25°C incubator and water was added every second day to prevent drying of the food. Eclosing flies were counted once a day in the afternoon for 9 days. Numbers of eclosing flies from the two successive vials (with same parental generation) were added together before analysing results. Therefore there are three data points for each individual cross in a lethality experiment. IBM SPSS Statistics (v.19) were used to quantify statistical differences using student's t-test. Each individual experiment was repeated twice.

4.2.2 Survival assay

The survival experiments were essentially life span experiments (see Sanz *et al*, 2010a), with some important differences. Three parallel crosses were made and 10-12 virgin females and 5-6 males

were used in each cross. Knockdown expressing flies were collected using CO_2 at the day of eclosion and kept in standard fly food vials, 10 flies per vial, males and females in separate vials. 30 flies of each genotype were collected. Flies were kept in a +25°C incubator with 12 h light-dark cycle. Flies were tipped into fresh vials every 2-3 days and dead flies were counted. The phenotype and activity of flies was also observed during the experiment. The experiment lasted two weeks, after which all vials were discarded. The percentage of living flies was recorded and survival curves were drawn using Microsoft Excell (2010). Each individual experiment was repeated twice.

4.3 Preparation and imaging of fly sections

4.3.1 Sectioning of paraffin embedded flies

Flies were fixed in 4 % formaldehyde for several hours (3-5 h). Prior to fixation the abdomen and wings were removed with dissection forceps (see 4.4.2). Fixed flies in cassettes were transferred to paraffin infiltration machine (Sakura Tissue-Tek VIP® 4). The paraffinization program used is illustrated in Table 4.4.

Table 4.4. Paraffin infiltration program. Reagent, concentration, time of incubation and temperature.

Reagent	Conc.	Time (h:mm)	Temp. (°C)
Ethanol	70 %	1:00	
Ethanol	80 %	1:00	
Ethanol	90 %	1:00	
Ethanol	100 %	1:30	
Ethanol	100 %	1:30	
Xylene		1:00	
Xylene		1:00	
Xylene		1:30	
Paraffin		1:30	58
Paraffin		1:00	58
Paraffin		1:00	58
Paraffin		1:00	58

Paraffin infiltrated flies were embedded in paraffin blocks individually, on a Sakura Tissue-Tek Embedding Console System. Flies were positioned so that the plane of cutting will be from dorsal to ventral side. Fly blocks were stored at +4°C.

4 μm slices were cut from fly blocks with Microm HM310 rotation microtome. Slices adhered to Superfrost plus microscope slides (Thermo Scientific) were left at +37°C overnight to dry completely and attach to the slides. Paraffin was removed from dry sections with xylene and a descending alcohol treatment, which is illustrated in Table 4.5.

Table 4.5. Deparaffinization protocol. Solution, concentration and time (in minutes).

Solution	Conc.	Time
Xylene		5
Xylene		5
Xylene		5
Ethanol	Abs.	Short
Ethanol	Abs.	Short
Ethanol	90 %	Short
Ethanol	90 %	Short
Ethanol	80 %	Short
Ethanol	80 %	Short
Ethanol	70 %	Short
Ethanol	70 %	Short
dH_2O		Short

4.3.2 Immunofluorescence staining and imaging with confocal microscope

Deparaffinated slides were rinsed with TBS-Tween before Heat Induced Epitope Retrieval (HIER) -treatment, in which samples were incubated for 30 minutes in +95°C citrate buffer. Citrate buffer was made dissolving 1.92 g anhydrous citric acid with 0.74 g EDTA into 1000 ml of distilled H_2O. The pH of solution was then adjusted to 6.2 and 0.5 ml of Tween 20 was added. The HIER-treated slides in the buffer were then cooled to room temperature (RT) on ice and slides were rinsed with TBS-Tween. Tissue sections were permeabilized by incubating them in 0.3 % Triton X-100 in TBS-Tween for 30 minutes in room temperature. Slides were washed after permeabilization with TBST (3 x 5 minutes). Sections were blocked with 5 % bovine serum albumin (BSA) in TBST overnight in +4°C. Primary antibodies were added in 5 % BSA in TBST solution and incubated for one hour at RT (list of primary and secondary antibodies in Tables 4.6 and 4.7). Primary antibody solution was washed away with TBST (3 x 10 minutes) before applying the secondary antibody cocktail, also in 5 % BSA in TBST solution and before incubation for one hour at RT. Slides were washed again with TBST (3 x 10 minutes) and a cover glass was mounted with ProLong Gold with DAPI (Invitrogen).

Table 4.6. List of primary antibodies. Antigen, antibody, manufacturer and dilution used.

Antigen	Antibody	Manufacturer	Dilution
COX IV	ab16056 Rb p	Abcam	1:200
ATP5A	ab14748 Ms m	Abcam	1:1000
AOX	PI047AB Rb p	21st century biochemicals	1:1000

Table 4.7. List of secondary antibodies. Antibody, manufacturer and dilution used.

Antibody	Manufacturer (cat #)	Dilution
Goat-anti-rabbit Alexa fluor 568	Invitrogen (A-11011)	1:1000
Goat-anti-mouse Alexa fluor 488	Invitrogen (A-10680)	1:1000

Slides were imaged using a Spinning Disc Confocal microscope (Microscope: Nikon Eclipse Ti, Confocal: Wallac-Perkin Elmer Ultraview, Camera: Andor EMCCD, Software: Andor iQ). Pictures were analysed with free software ImageJ (v. 1.45s) downloadable from the internet.

4.3.3 Hematoxylin and eosin staining and imaging with light microscope

Deparaffinated slides of fly tissues were stained in hematoxylin (Mayers, Merck, diluted 1:4 with dH_2O) for 3 minutes, then washed with running tap water for 10 minutes. Washing was followed by staining in 0.1 % Eosin Y (Histola, glacial acetic acid added) for 40 seconds. Excess colour was quickly rinsed off with dH_2O. Before mounting the cover slide with organic mounting medium (EUKITT), slides were cleared with an ascending ethanol series and xylene (same as in Table 4.5 but in reverse order).

Images of the slides were taken with an Olympus BX 51 microscope attached with an Olympus ColorView IIIu camera. The camera was operated via Olympus CellB software v. 2.4. Pictures were analysed with free software ImageJ (v. 1.45s) downloadable from the internet. Sequential pictures of fly brain were aligned using StackReg plugin for ImageJ.

4.4 Characterization of elav-GAL4 driver expression pattern

2-3 parallel crosses were made in both mating chambers and standard fly food vials. 24 or 36 virgins and 12 or 18 males, respectively, were used for crosses in mating chambers and 12 virgins and 6 males were used for crosses in standard fly food vials. Flies were left to copulate in mating chambers for one day before starting the experiment. Microscope system used for imaging:

Olympus SZX16 microscope, Olympus ColorView IIIu camera, Olympus Cell^{B} software (v. 2.4), Olympus U-RFL-T fluorescent lamp.

4.4.1 Collection of fly embryos and first instar larvae

The mating of flies was carefully timed, in order to collect embryos at a certain stage of development. A fresh plate (60 x 15 mm, Greiner bio-one) of fly food was added to the bottom of the mating chamber every two hours. Mating chambers and plates with embryos were stored at +25°C during the experiment. After 14-16 hours post-copulation (and assumed fertilization), embryos were collected from the petri dish/plate. The chorion was removed from embryos prior to microscopy by rolling the eggs on double sided tape. Dechorionated embryos were placed on standard microscope slides and a drop of 70 % glycerol was added on top of the embryos to prevent drying prior to microscopy.

First instar larvae were collected from plates 24-36 hours post-copulation. Larvae were rinsed in PBS and dried on tissue. Clean larvae were placed on +4°C microscope slides (Thermo-Scientific, Superfrost plus) and covered with 70 % glycerol before placing a cover slip carefully on top of them. Larvae were immobilized prior to microscopy by placing them at −20°C for 10 minutes.

4.4.2 Dissecting flies at various stages of development

All dissections were done with Dumont #55 INOX forceps while the dissectible objects were submerged in PBS.

4.4.2.1 Dissection of third instar larvae

Third instar larvae were collected from plates 72-84 h after copulation. The posterior end of larva was cut away using forceps, which extrudes the internal organs of the larva. The larvae were grabbed by the mouth hooks and rolled inside out on top of the forceps. This way the central nerve cord, gut and other tissues were easily collectable. Larval muscles were pictured whilst still attached to cuticle. Dissected tissues were left submerged in PBS when taking pictures.

4.4.2.2 Dissection of pupae

Pupae were collected for dissection from vials when the eye pigmentation was visible through the pupal case (stages P8-P9). Pupae were attached to microscope slide with double sided tape, ventral side down (same way as attached to the vial). The pupal case was opened from the anterior end

where the fly would eclose naturally. The puparium was then cut carefully toward the posterior end and loose parts of the pupal case were lifted away. After three quarters of the pupal case was removed, forceps were pushed under the exposed fly which then became attached to the forceps. The fly was then removed from the puparium. A drop of 70 % glycerol was added to prevent the fly from drying. An exposed fly was then pictured at this stage.

The fly exposed from puparium was cut from the posterior end of the abdomen on the dorsal side. A small volume of PBS was gently pipetted back and forth to remove all loose cells from abdomen and thorax. The cuticle was then ripped from the posterior to the anterior end, on the dorsal side, to expose soft tissues for dissection. Thoracic muscles were removed at this point to expose the central nerve cord. The central nerve cord was lifted from the ventral side, using forceps, while it was still attached to the brain. After that, the brain from the head was cleaned clear of other tissues (especially trachea).

4.4.2.3 Dissection of adult flies

Flies were imaged and dissected 1 day and 5-6 days after eclosion. Adult flies were placed in an empty vial with a cotton swab soaked in FlyNap (Carolina Biological Supply Company). After 10-20 min unconscious flies were collected for imaging.

Dissection of adult flies started by removing the wings and legs, then the abdomen. Brains were exposed by first removing proboscis, then cutting both compound eyes out and ripping the cuticle of the head to remove it, starting from the anterior. When the brain was carefully exposed, excess tissues attached to it were removed. Trachea was entirely removed as it interferes with fluorescent imaging. The thorax was cut from both sides and then the dorsal half was raised to expose the central nerve cord, attached on the ventral side. Muscles were then removed from the dorsal side of the thorax. The central nerve cord was removed whilst still attached to the brain by carefully sliding forceps between the central nerve cord and the cuticle and raising the nervous tissue. Once the central nerve cord was loose from the ventral thoracic cuticle it was cleaned as other tissues.

Internal organs are in the abdomen and those of interest were cut away by first cutting the abdominal cuticle from the lateral sides. Once the cuticle was removed, all organs of interest were easily accessible.

5. RESULTS

5.1 Whole organism phenotypes resulting from tissue specific knockdown of COX subunits

As stated before, Kemppainen *et al* (unpublished) performed a preliminary experiment which showed that COX subunit knockdown in the *Drosophila* nervous system failed to produce any phenotype. To study the effects of nervous system -restricted COX knockdown more specifically, three nervous system -specific GAL4 drivers were used to drive two RNAi constructs, against COX IV and COX Vb subunits. The effects on the phenotype using each driver-RNAi pair were recorded using lethality and survival assays. AOX coexpression was then used to attempt to rescue flies with deleterious phenotypes.

Crosses for determining the lethality of neural specific COX knockdown are presented in Appendix 1A. Homozygous UAS-RNAi females were crossed with either heterozygous GAL4 driver males or hemizygous GAL4 driver males, depending on the driver. Wild-type females were crossed with each GAL4 driver male as a control. Crosses to determine whether AOX can rescue lethality due to neural specific COX knockdown are illustrated in Appendix 1B. Crosses for determining viability of neural specific COX knockdown are presented in Appendix 1C and the respective crosses for determining viability of AOX rescued neural COX knockdown flies are presented in Appendix 1D.

Student's t-test was used to evaluate statistical significance between knockdown flies and controls in lethality assays. However, because the number of repeats is 3 in all cases of performed lethality assays, reliability of the statistical tests is questionable. Although statistical significances are marked in the figures, they should not be considered as definitive. However, in this case the descriptive data on the phenotype is more important. In survival assays the statistical program GraphPad Prism was unable to make reliable log-rank tests on survival curves because most of the flies in the experiments survived the two week surveillance period (therefore they have to be presented as 0 in the data which means that they are censored from the original input data). Descriptive data collected during the survival assay is therefore very important.

5.1.1 Neural specific knockdown using Nrv2-GAL4 driver has no effect on phenotype

Neuronal knockdown of COX subunits was tested with an Nrv2-GAL4 driver that was previously tested by Kemppainen *et al* (unpublished). In that experiment, knockdown of COX Vb and COX VIb did not affect the viability of flies. I repeated this experiment, this time with RNAi against COX IV and COX Vb. The Nrv2-GAL4 driver was balanced over CyO balancer chromosome (see

materials and methods 4.1.1). Once again, Nrv2-GAL4 driven COX knockdown did not affect the viability of flies (Figure 5.1). There was a slight difference between the numbers of eclosing COX IV knockdown flies and wild-type flies, in both sexes, but it was within standard deviation. There was no statistical difference between knockdown flies and wild-type flies, in either sex, or when compared to internal controls (flies with balancer chromosome). The phenotype of the knockdown expressing flies was identical to wild-type at the day of eclosion, meaning that flies were flying and jumping actively around the vial.

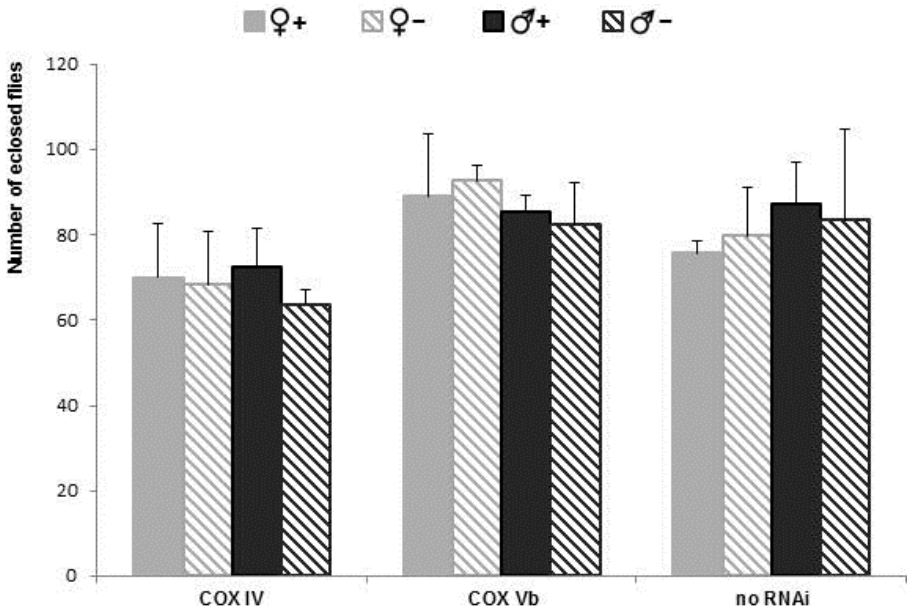

Figure 5.1. Mean number of eclosed flies after Nrv2-GAL4 driven COX subunit knockdowns. Plus sign in the legend denotes flies with Nrv2-GAL4 driver and a minus sign denotes flies without the driver (flies with balancer). Means are presented ± SD. No statistical differences were observed. See Appendix 1 for further details of crossing scheme.

The effect of Nrv2-GAL4 driven COX subunit knockdown on degeneration of flies was tested with a survival assay. The experimental set-up differed from the lethality assay, so that all progeny flies were expressing the RNAi constructs (see crosses in Appendix 1). During the two-week surveillance period, the phenotype of knockdown flies was the same as that of wild-type flies. Knocking down either COX subunit did not seem to have any degenerative effect (Figure 5.2). A few COX Vb knockdown male flies died during the experiment but 90 % stayed alive. A few deaths also occurred of wild-type female and male flies, so the observed attrition might be considered natural variation.

Since Nrv2-GAL4 driven knockdown was not able to produce any kind of phenotype, the driver was discarded from further experiments.

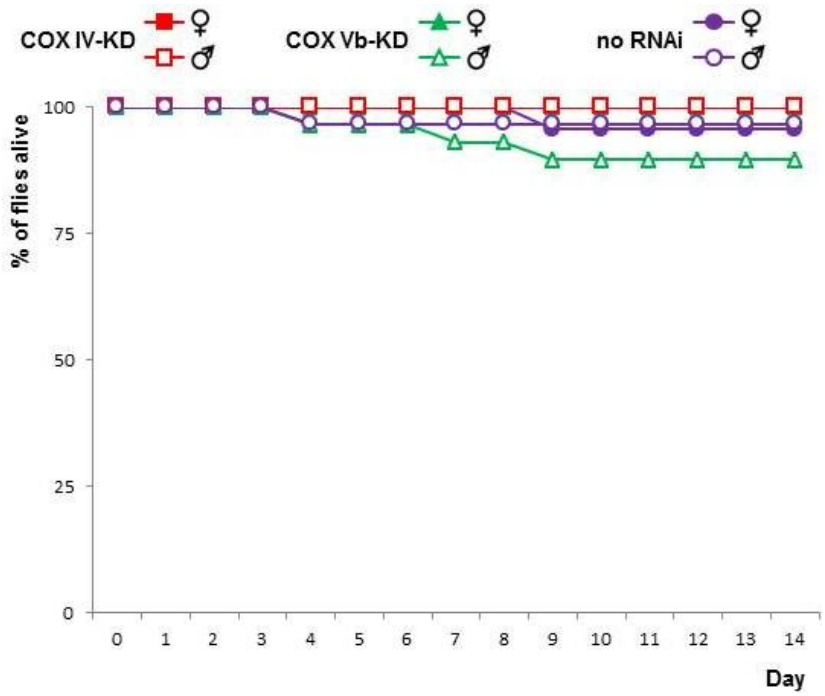

Figure 5.2. Survival of flies when COX subunits were knocked down using Nrv2-GAL4 driver. More than 90 % of flies from all genotypes survive the experiment period. The number of flies in the experiment was 30 for COX IV-KD males, COX IV-KD females and wild-type male flies, 29 for COX Vb-KD females and COX Vb-KD males and 23 for wild-type females.

5.1.2 Neuronal knockdown using 3rd chromosomal elav-GAL4 driver

5.1.2.1 Knocking down COX IV subunit using elav(3)-GAL4 produces a partially lethal phenotype

Elav(3)-GAL4 driver (Bloomington stock centre number 8760) was also tested in the preliminary experiment of Kemppainen *et al* (unpublished). At the time, it did not produce a phenotype when it was driving knockdown of COX subunits Vb and VIb. The test was repeated, and for this elav(3)-GAL4 driver was balanced over Sb balancer chromosome (see materials and methods 4.1.1). A lethality assay was performed, which verified that elav(3)-GAL4 driven knockdown of COX subunit Vb did not produce a lethal phenotype in flies (Figure 5.3). Knockdown flies had the same phenotype as wild-type flies at the day of eclosion: they were active, flying and jumping around the vial. However, when the elav(3)-GAL4 driver was used to drive knockdown of COX IV, a partially lethal phenotype was produced. Most of the eclosing flies dropped from the pupa to the bottom of the vial and got stuck on the fly food. They were not able to move or get out from the food so most of them died in first few hours after eclosion. In addition to the flies with “weak” phenotype, there were a few flies eclosing that had a wild-type locomotor phenotype, but they were the minority.

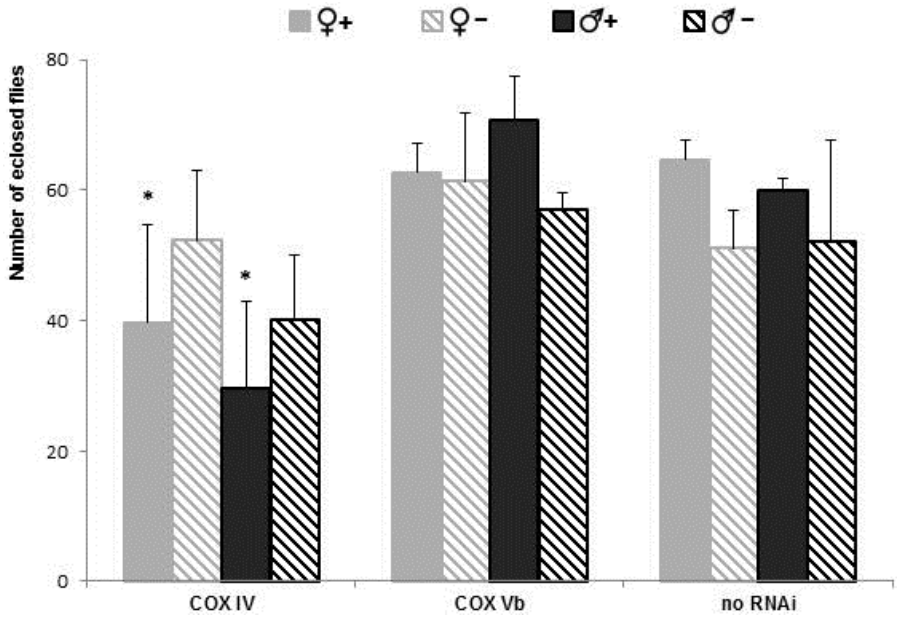

Figure 5.3. Mean number of eclosed flies when COX subunit knockdown is driven with elav(3)-GAL4 driver. Number of individual repeats for each genotype is 3 (as it was with Nrv2-GAL4 driven lethality assay). Mean numbers are presented ± SD. Plus sign in the legend denotes flies with elav(3)-GAL4 driver and a minus sign denotes flies without the driver. An asterisk represents statistically significant difference when the mean values are compared to those of wild-type flies ($p<0.05$, Student's t-test). No statistically significant difference was observed if number of eclosed knockdown flies was compared to number of internal control flies.

5.1.2.2 Knocking down COX IV subunit using elav(3)-GAL4 degenerates flies

Because COX subunit Vb knockdown did not produce any phenotype, only COX IV knockdown flies were collected for the survival assay. Flies were collected from a cross between homozygous *UAS-RNAi* females and homozygous *elav(3)-GAL4* –males (see Appendix 1 for crossing scheme). As a control, wild-type females were crossed with homozygous *elav(3)-GAL4* –males. Some eclosing COX IV knockdown flies got stuck in the food, as in the lethality assay, but these were not collected for the survival experiment. Both male and female flies collected for the experiment were weak compared to wild-type flies at the day of eclosion. Half of the knockdown males died one day into the experiment, as can be seen from Figure 5.4. After two weeks only 21 % of male knockdown flies were alive. Knockdown female flies did not die as frequently as male knockdown flies, 88 % were still alive after two weeks. Both female and male knockdown flies were immobile for the two weeks. They did not fly or move even when the vial was picked up. Both males and females preferred lying still on the wall of the vial. However, if they dropped to the bottom of the vial, they were able to climb back up to the wall. All wild-type females and males survived the two week period.

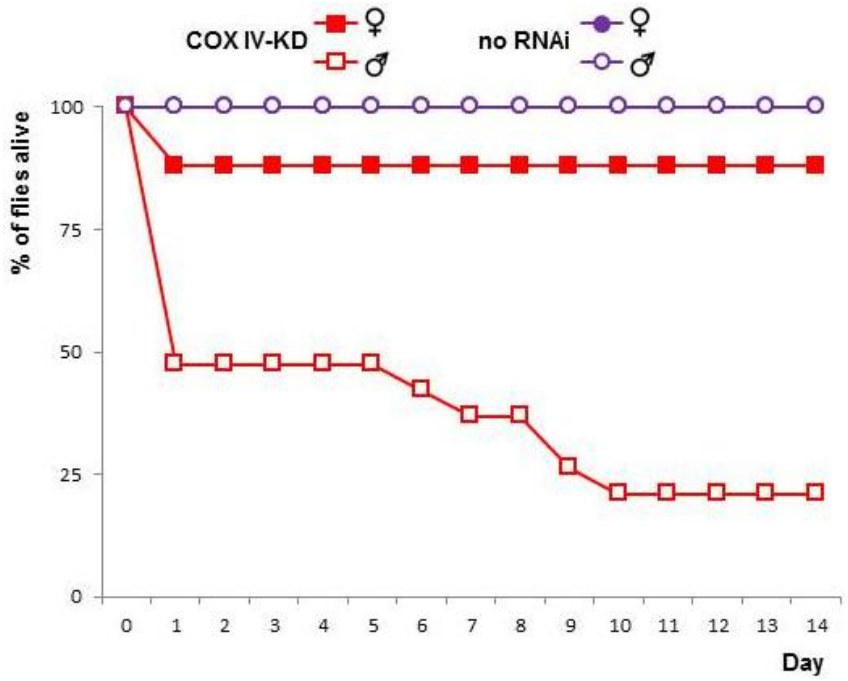

Figure 5.4. Survival of flies when COX subunit IV was knocked down using elav(3)-GAL4 driver. All wild-type flies, both male and female, survived through the two week experiment. The number of flies in each group was 29, 19, 30 and 30 for COX IV-KD female, COX IV-KD male, wild-type female and wild-type male, respectively.

5.1.3 Neuronal knockdown using X-chromosomal elav-GAL4 driver

5.1.3.1 Knocking down COX subunits by elav(X)-GAL4 produces lethal phenotypes

A third neuron-specific driver, X-chromosomal elav-GAL4, was chosen to drive the expression of transgenes because it was postulated to be more efficient than the third chromosomal driver. Because *Drosophila* males carry only one X-chromosome, the experimental crosses were performed so that only female offspring express the knockdown and male offspring act as a control group for the females (see crosses in Appendix 1).

Only a few COX IV knockdown females were able to eclose in the experiment (Figure 5.5). The eclosing females were extremely weak: they fell to the bottom of the vial and were unable to move. They were unable to inflate their wings. All these females died soon after eclosion. The respective control males had the same phenotype as wild-type controls, they were flying and jumping around the vial actively. The phenotype of elav(X)-GAL4 driven COX IV knockdown females resembled the phenotype of elav(3)-GAL4 driven COX IV knockdown flies. Knocking down COX Vb using elav(X)-GAL4 also produced a semilethal phenotype. There were significantly less females eclosing than males. However, knocking down COX subunit Vb did not change the phenotype of the flies that reached eclosion: knockdown females were active and flying around the vial just as much as wild-type flies.

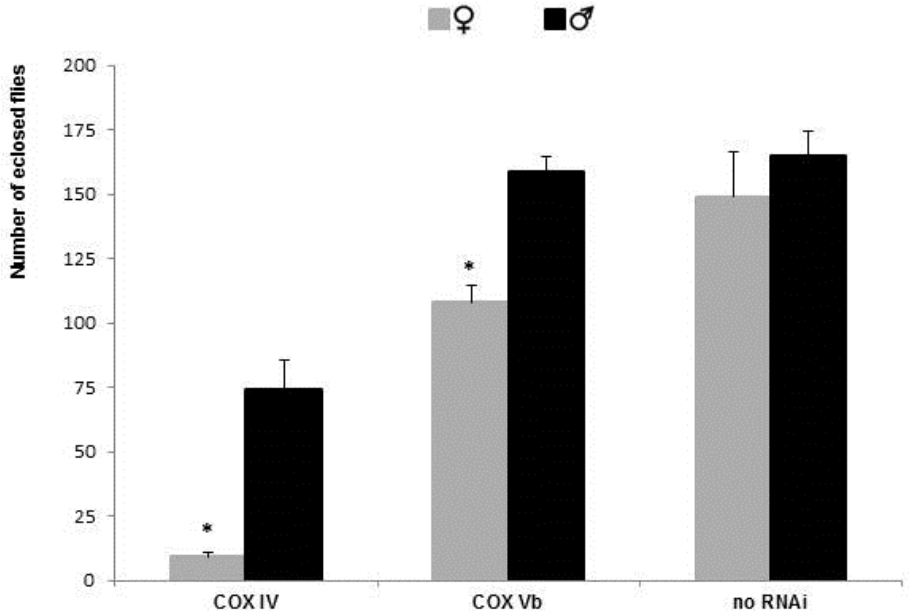

Figure 5.5. Mean number of eclosed flies when COX subunit knockdown was driven using elav(X)-GAL4 driver. In this experiment female flies are expressing the knockdown and males are an internal control. Mean numbers are presented ± SD. An asterisk represents statistically significant difference when knockdown females are compared to both wild-type females and respective control males ($p<0.05$, Student's t-test).

5.1.3.2 Knocking down COX subunit Vb by elav(X)-GAL4 produces a degenerative phenotype

The survival assay for flies knocked down for COX IV or COX Vb using the elav(X)-GAL4 driver was again set up in such a way that all the offspring were expressing the RNAi construct (see Appendix 1). No viable COX IV knockdown flies (of either sex) eclosed, so the measurement could not be performed on these flies. COX Vb knockdown produced a strong phenotype in males, but less strong in females, which manifested the same phenotype as wild-type flies at the day of eclosion. Eclosing knockdown males were very weak. Most dropped to the bottom of the vial and got stuck in the food. They had essentially the same phenotype as COX IV-KD females. Some male flies were less severely affected and were transferred to new vials with fresh fly food for the survival experiment. These males were still very weak and preferred lying still on the wall of the vial. One week into the experiment, the male flies started to die off and after two weeks only 10 % were alive (Figure 5.6). COX Vb-KD females appeared normal on the day of eclosion but they quickly turned immobile and remained still on the wall of the vial. They were slow in getting back up if they dropped to the bottom of the vial. However, more than 90 % of the females survived the two week period, even though their phenotype was severely affected due to the knockdown.

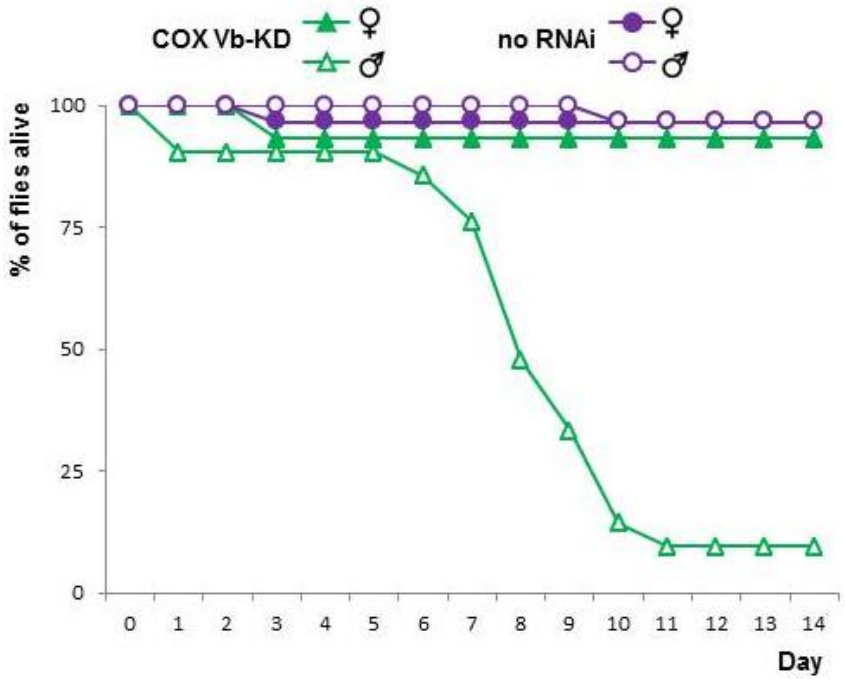

Figure 5.6. Survival of flies when COX subunit Vb is knocked down with elav(X)-GAL4 driver. Number of flies in each group were 30, 21, 30 and 30 for COX Vb-KD females, COX Vb-KD males, wild-type females and wild-type males, respectively.

If the phenotype of the flies is severely affected by COX knockdowns, is the overall morphology of brain also affected due to the neural specific COX subunit knockdown? To answer this question knockdown expressing females were collected from lethality experiment and casted into paraffin blocks (see materials and methods section 4.3.1). Fly brains were sequentially sliced and then stained with hematoxylin and eosin for better view of tissue structures. In Figure A2.1 (Appendix 2) is a representative slice from both knockdown expressing brain and wild-type control brain. No apparent change in the overall morphology of the brain could be distinguished. It seems reasonable that flies that make it through the metamorphosis and out of the pupae have normally developed brain, even if they are expressing COX knockdown. If the knockdown would somehow disturb the formation and maturation of central nervous system, it would probably lead to death at earlier stages of development.

5.2 AOX rescue of whole-organism phenotypes resulting from neuronal specific COX knockdown

5.2.1 AOX rescue of flies knocked down for COX IV using elav(3)-GAL4

5.2.1.1 AOX rescues lethality of COX IV knockdown driven by elav(3)-GAL4

Knocking down COX subunit Vb with elav(3)-GAL4 failed to produce any phenotype so AOX rescue experiments were not performed for these flies. Flies for the COX IV knockdown lethality experiment were collected from a cross between homozygous RNAi females with or without a homozygous AOX transgene and homozygous elav(3)-GAL4 males. A more detailed crossing scheme is presented in Appendix 1.

AOX coexpression made no difference to lethality (Figure 5.7). In each group more male flies eclosed than females. However the numbers do not provide the whole picture. COX IV knockdown flies were weak as in the preceding elav(3)-GAL4 driven lethality assay. Most of them got stuck in the fly food at the bottom of the vial and died. The ones that avoided getting stuck on the fly food lay still on the wall of the vial. AOX coexpressing flies on the other hand were active and were able to fly and jump around the vial on the day of eclosion. There were still some flies that got stuck in the food and died, but considerably less than in vials with flies expressing only COX IV-KD.

To confirm that the possible rescue of lethal phenotype results from AOX coexpression, alternative NADH dehydrogenase (Ndi1) was also expressed in flies with RNAi constructs. Ndi1 is a bypass enzyme of complex I, therefore it should not have effect on COX knockdown (Sanz *et al*, 2010b). Ndi1 coexpressing flies were even more affected than flies with COX IV knockdown alone. All of the eclosing COX IV-KD;Ndi1 flies fell to the bottom of the vial and died.

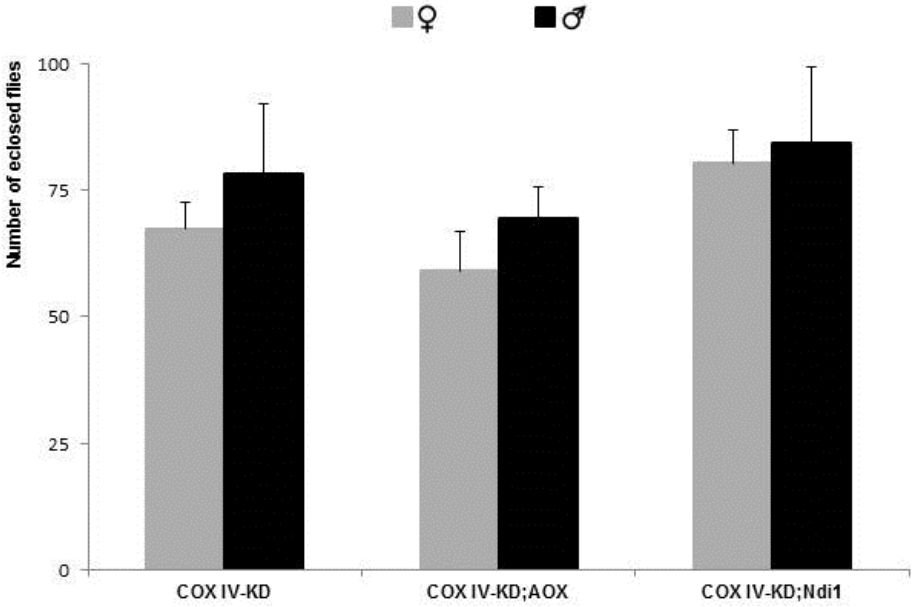

Figure 5.7. Mean number of eclosed flies with COX subunit IV knockdown coexpressing AOX or Ndi1 when expression is driven with elav(3)-GAL4. Flies express both knockdown of COX IV and a transgene illustrated in the X-axis. All eclosing flies are expressing knockdown and transgene if present. Statistically significant differences were not observed.

5.2.1.2 AOX partially rescues decreased viability in flies with COX IV knockdown driven by elav(3)-GAL4

Survival of neural specific COX IV knockdown flies rescued with AOX is presented in Figure 5.8. Flies coexpressing AOX survived longer than flies expressing just the knockdown. Survival percentages after 14 days were 100 % and 88 % for female COX IV-KD;AOX and COX IV-KD flies, respectively, and for males 81 % and 21 %, respectively. Flies expressing only the knockdown (both males and females) were weak in phenotype: they were standing still on the wall of the vial and did not fly or run around. They could climb back up the wall if they dropped to bottom of the vial. However, their movement was slow and they seemed to prefer lying still. This phenotype remained the same during the two week experiment. AOX coexpressing flies not only survived longer but there was a major difference in their phenotype compared to flies knocked down for COX IV. AOX coexpressing flies (of either sex) were actively flying and jumping around the vial. They behaved essentially as wild-type flies. Only a few deaths occurred among the COX IV-KD;AOX males, but the remaining stayed active throughout the two weeks. There was also no change in the phenotype apart from the deaths.

In the lethality experiment (see results 5.2.1.1) Ndi1 coexpression appeared to make flies even weaker than when they were knocked down for COX IV. COX IV-KD;Ndi1 flies also appeared weaker in the survival assay and died faster than flies knocked down for COX IV only (Figure 5.8).

73 % and 6 % of the COX IV-KD;Ndi1 female and male flies survived the two week experiment, respectively. The general phenotype of the flies was the same as flies expressing knockdown only. They were not flying or moving but remained still on the wall of the vial. They could, however, also climb the wall of the vial if they dropped to the bottom.

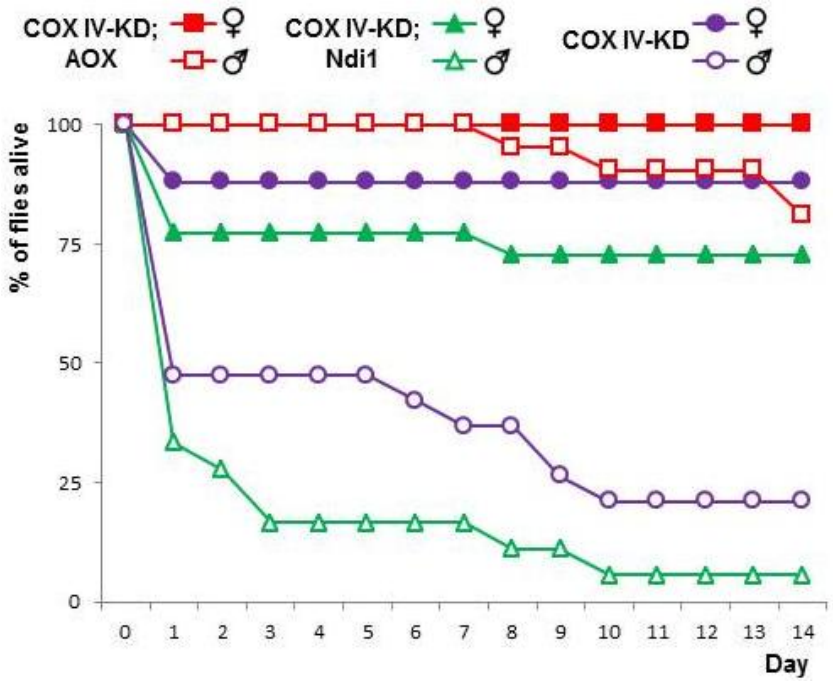

Figure 5.8. Survival of flies when elav(3)-GAL4 driven COX IV knockdown was rescued with AOX. Flies coexpressing AOX survived longer than flies with knockdown only. Flies coexpressing Ndi1 died faster than flies with knockdown only. This was the case for both sexes. The number of flies in the experiment in each group was 8, 21, 22, 18, 25 and 19 for COX IV-KD;AOX female, COX IV-KD;AOX male, COX IV-KD;Ndi1 female, COX IV-KD;Ndi1 male, COX IV-KD female and COX IV-KD male, respectively.

5.2.2 AOX rescue of flies with COX knockdown driven by elav(X)-GAL4

5.2.2.1 AOX rescues lethality of flies with COX knockdown driven by elav(X)-GAL4

In the X-chromosomal elav-GAL4 driven lethality assay, female progeny were expressing transgenes, whereas male flies act as an internal control (see crossing scheme in Appendix 1). As an additional control, lethality assay was performed on flies expressing both RNAi construct against COX Vb and GFP. The results are presented in Appendix 3.

As in the previous elav(X)-GAL4 driven lethality assay (results section 5.1.3.1), COX I-KD female flies were extremely weak and most died immediately after eclosion on the bottom of the vial. Some flies were unable to eclose from the pupae. Also there were fewer male flies eclosing than in other genotypes as was the case in the previous assay. This might be due to the fact that *COX IV-RNAi* homozygous females are poor at laying eggs. Although AOX coexpression seemed to

partially rescue the lethal phenotype (Figure 5.9), the eclosing females were still extremely weak and died immediately after eclosion. The difference in lethality is caused by the fact that there were more female flies surviving through pupal stage to eclosion. Some of the AOX-rescued females were also able to inflate their wings, something that no COX IV knockdown females could do. Ndi1 coexpressing females were equally weak as female flies expressing only COX IV knockdown.

Approximately half of the eclosing COX Vb-KD females looked normal at the day of eclosion, i.e. they had a wild-type phenotype. However, the other half of eclosing female flies became stuck in the food at the bottom of the vial and died. COX Vb knockdown produced a semi lethal phenotype as in the previous experiment (Results 5.1.3.1). AOX coexpression rescues this semi lethal phenotype. Most of the eclosing females had a wild-type phenotype on the day of eclosion but there were still a few flies that got trapped in the food and died. On the other hand, Ndi1 coexpression seemed to make knockdown flies even weaker. There was roughly the same number of female flies eclosing from COX Vb-KD and COX Vb-KD;Ndi1 vials, but the majority of females coexpressing Ndi1 became stuck in the food after eclosion and died soon thereafter.

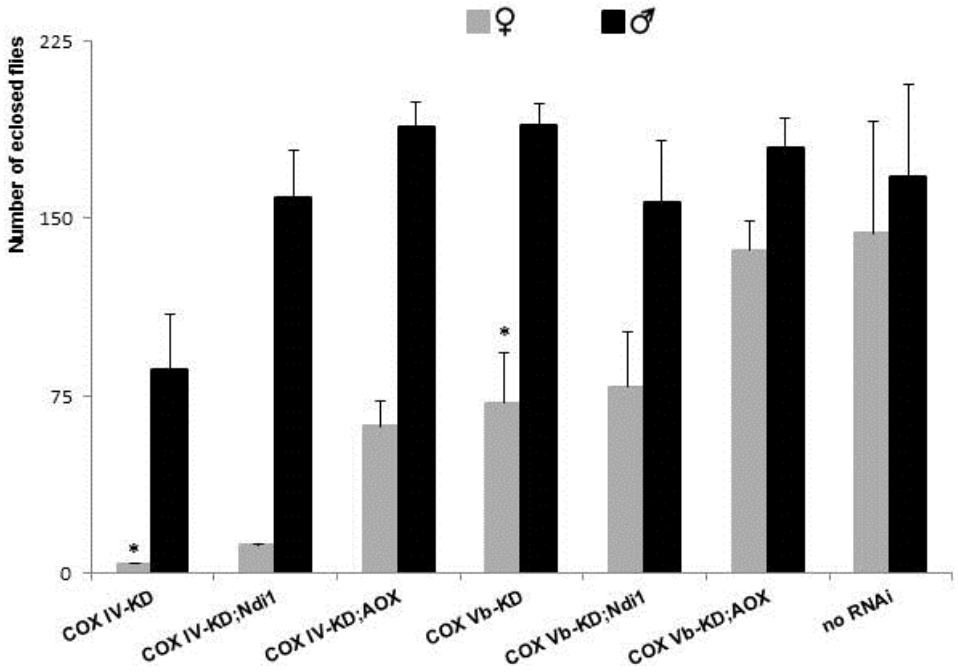

Figure 5.9. Mean number of flies eclosed when elav(X)-GAL4 driven COX knockdown was rescued with AOX. Female flies are expressing the knockdown and males are internal controls. Mean numbers are presented ± SD. In statistical analysis both COX subunit knockdown females were compared to respective males and wild-type females as well as AOX females with same COX subunit knockdown. *=$p<0.05$, Student's t-test.

5.2.2.2 AOX prevents degeneration of flies knocked down for COX by elav(X)-GAL4

In the survival experiment all of the offspring were expressing both knockdown and transgenes (see crosses in Appendix 1). COX IV knockdown was lethal even with AOX coexpression. All of the flies got stuck on the food and died. Many COX IV-KD flies got stuck in the pupal case and could not eclose. A representative picture of flies stuck in the fly food and flies unable to eclose is presented in Appendix 2 (Figure A2.2). This resembles a phenotype derived from muscle specific knockdown of COX (Kemppainen *et al*, unpublished). There were no males eclosing in COX IV-KD vials or COX IV-KD;Ndi1 vials. However males did eclose in COX IV-KD;AOX vials but they are extremely weak and died soon. Thus flies with COX IV knockdown could not be collected for a survival experiment. The experiment was therefore performed only on flies knocked down for COX Vb (and/or expressing other transgenes).

The same pattern of survival was seen as in the survival experiment with elav(3)-GAL4 driven COX IV knockdown in section 5.2.1.2. Female flies survived longer than the corresponding males. AOX coexpression rescued the decreased survival caused by COX Vb knockdown. Ndi1 coexpression with COX Vb-KD causes flies to die faster (Figure 5.10). Males expressing COX Vb knockdown started to die seven days into the experiment and only 10 % survived the whole two weeks. Males coexpressing Ndi1 with COX Vb knockdown all died within a week. Both COX Vb-KD and COX Vb-KD;Ndi1 males remained still on the wall of the vial, although they were still able to fly and jump. Their movement appeared “shaky” compared to wild-type flies or those coexpressing AOX. This “shaky” movement was recorded in a video. Males coexpressing AOX had a wild-type phenotype i.e. they jumped and flew as much as wild-type flies. 93 % of these flies survived the two-week period.

COX Vb-KD and COX Vb-KD;Ndi1 females had essentially the same phenotype as their male counterparts described above. 93 % and 50 % of these flies survived the two-week experiment, respectively. COX Vb-KD;AOX females were very active. There was no difference when they were compared to wild-type flies. All COX Vb-KD;AOX females survived the experiment.

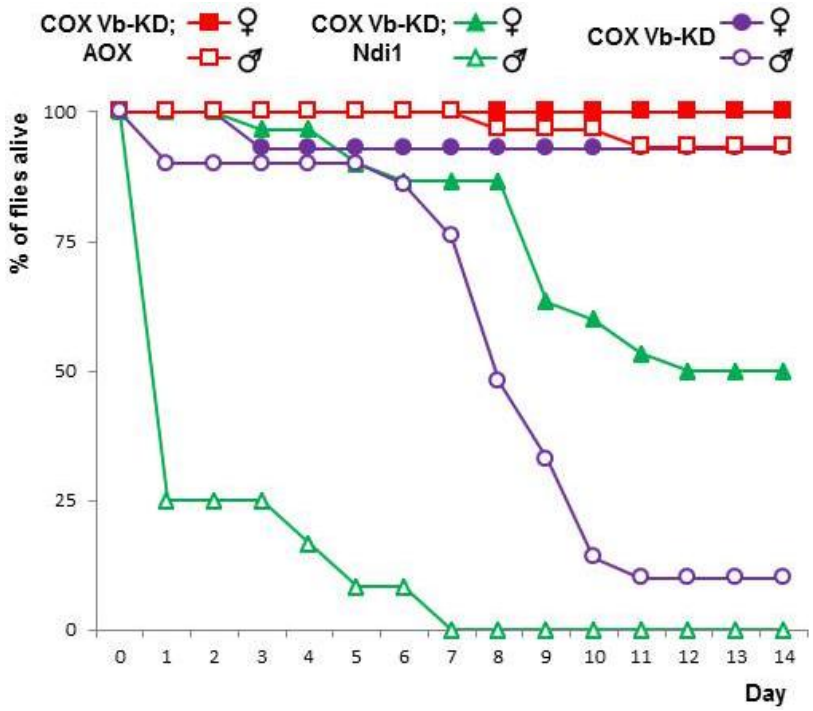

Figure 5.10. Survival of flies when elav(X)-GAL4 driven COX Vb knockdown is rescued with AOX. The number of flies in each group was 30, 30, 30, 12, 30 and 21 for COX Vb-KD;AOX females, COX Vb-KD;AOX males, COX Vb-KD;Ndi1 females, COX Vb-KD;Ndi1 males, COX Vb-KD females and COX Vb-KD males, respectively.

5.3 Tissue specific UAS-AOX expression and knockdown of COX using the X-chromosomal elav-GAL4 driver

In order to verify the functionality of the knockdown, the presence of COX subunits was detected from fly brain and muscle sections by staining with antibodies. Only the X-chromosomal elav-GAL4 driven transgene expression was checked, as it produced the strongest phenotype. AOX expression in fly brain and muscle are presented in Figures 5.11 and 5.12. AOX was present in the brain when it was coexpressed under conditions of COX subunit knockdowns. Staining of muscle for AOX was not expected to give any signal because the driver used was supposedly neuron-specific. However, AOX staining is clearly visible in Figure 5.12, showing that thoracic muscles of the fly were coexpressing AOX upon COX subunit knockdown. Elav(X)-GAL4 driver therefore seems to have some activity in thoracic muscle of the adult fly.

Representative immunocytochemistry images showing COX knockdown efficiency are presented in Appendix 4 (Figures A4.1 and A4.2).

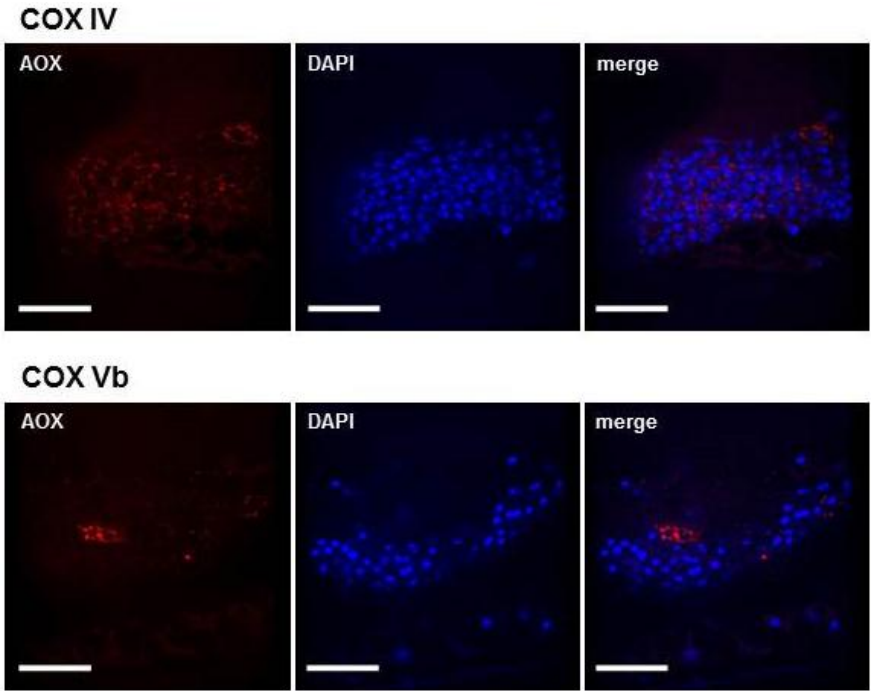

Figure 5.11. Expression of AOX in fly brain when driven by X-chromosomal elav-GAL4 driver. AOX is expressed in flies knocked down for COX IV or COX Vb. Scale bar 20 µm.

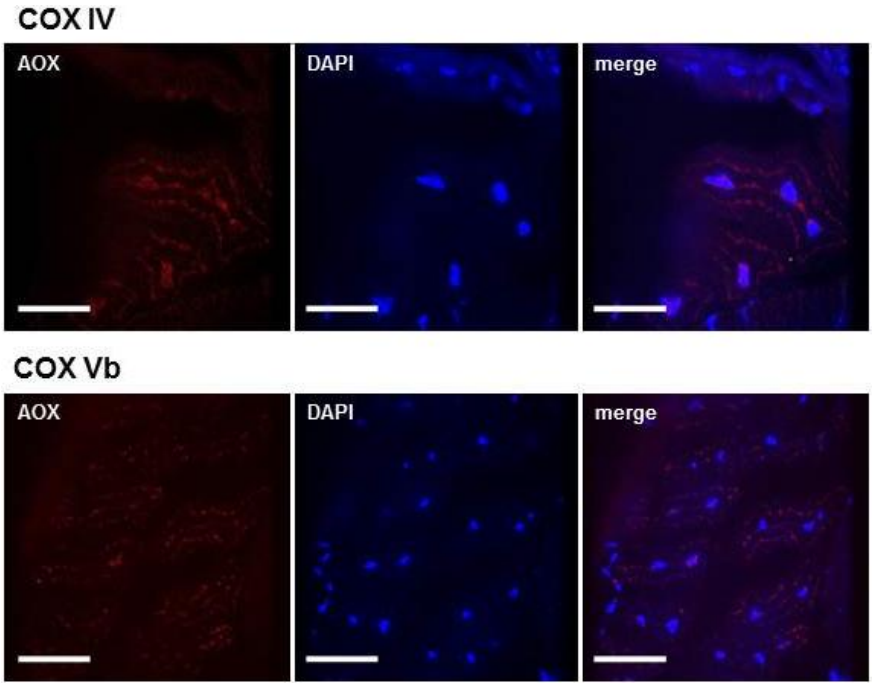

Figure 5.12. Expression of AOX in fly muscle when driven by X-chromosomal elav-GAL4 driver. Scale bar 20 µm.

5.4 Characterization of neural GAL4 drivers by UAS-GFP expression pattern -assay

The phenotype of flies with COX IV or COX Vb knockdown, driven with either elav(3)-GAL4 or elav(X)-GAL4, resembled to some extent that of flies with a muscle-specific COX knockdown. Flies became sessile progressively and were barely able to climb back up the wall of the vial if they

dropped to the bottom. Also, their movement was slow and appeared shaky. Together with the findings from AOX immunocytochemistry (section 5.3) this suggests that the phenotypes may be due partly (or wholly) to COX knockdown in muscle). In order to find out the extent of driver "leakiness", homozygous elav-GAL4 -driver females were crossed with homozygous UAS-GFP males (see crosses Appendix 1E). The mating chambers used in the crosses were covered because darkened surroundings encourage females to lay more eggs. The GFP expression pattern was recorded microscopically at all stages of development and the non-translucent stages were further dissected to test for GFP expression in different tissues.

The results of GFP expression pattern analysis are presented in figures 5.13-5.18 for both elav-GAL4 drivers. GFP was expressed in equal amounts using either driver and in the same tissues and corresponding developmental stages. In late stage embryo and in L1 larvae, GFP was expressed only in the central nervous system and the peripheral nerve bundles (Figure 5.13). Expression of GFP in third instar larvae is presented in figures 5.14 and 5.15 for elav(X)-GAL4 and elav(3)-GAL4, respectively. At this stage, the central nervous system showed high expression of GFP but both drivers seem to support GFP expression also in the salivary glands. However, there is no GFP expression in the muscle of the larvae. Expression in pupal stage differed slightly between the two drivers (Figure 5.16). CNS showed high expression of GFP when driven by both elav-GAL4 drivers but muscle seemed to express GFP only when driven with elav(X)-GAL4. No GFP expression in muscle could be detected in pupae when expression was driven with elav(3)-GAL4. In adult flies the GFP expression pattern was similar between the two drivers (Figures 5.17 and 5.18). There was high expression of GFP in the central nervous system and also low expression in muscle. GFP was also expressed in other tissues, for example in the testis of male flies and in some parts of the digestive system. GFP expression pattern of muscle specific driver G14-GAL4 are presented for comparison in figure 5.19. The flies shown in the various images were all expressing nuclear targeted GFP ("stinger": Barolo *et al*, 2000) unless otherwise stated.

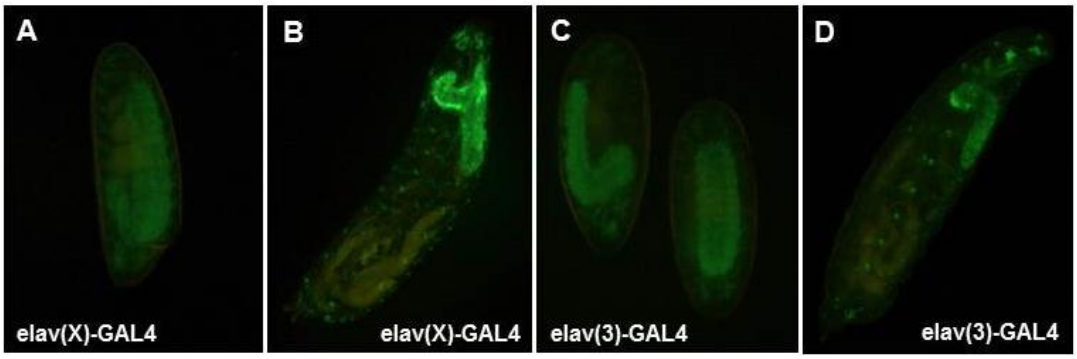

Figure 5.13. GFP expression in embryo and L1 larvae driven by elav-GAL4 drivers. Embryos pictured here are at stage 16 (14-16 h after fertilization). GFP expression driven with elav(X)-GAL4 is presented in A) in which the embryo is positioned anterior down (ventrolateral view) and in B) in which the first instar larva is positioned anterior up (dorsolateral view). GFP expression driven with elav(3)-GAL4 is presented in C) in which the two embryos are both positioned anterior down. View in the left embryo is lateral, in the right embryo it is ventral. In D) the first instar larva is positioned anterior up (lateral view). Fly food in the gut of larvae is autofluorescent.

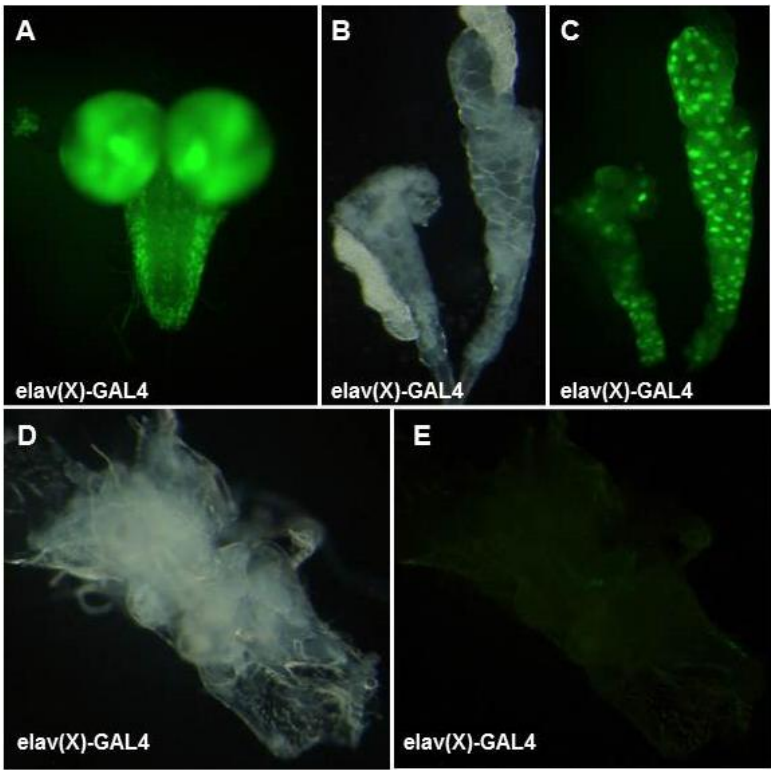

Figure 5.14. GFP expression in third instar larval tissues driven by elav(X)-GAL4. A) Brain and ventral nerve cord, dorsal view. Salivary glands are pictured under B) visible light and C) fluorescence illumination. Muscles of the larva, still attached to the cuticle, are pictured under D) visible light and E) fluorescence illumination.

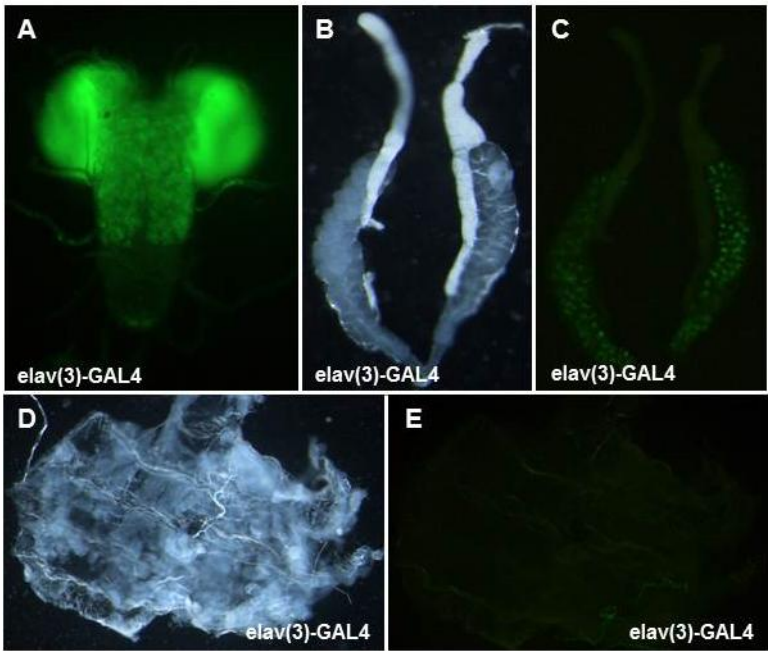

Figure 5.15. GFP expression in third instar larval tissues driven by elav(3)-GAL4. A) Brain and ventral nerve cord, ventral view. Salivary glands are pictured under B) visible light and C) fluorescence illumination. Muscles of the larva, still attached to the cuticle, are pictured under D) visible light and E) fluorescence illumination.

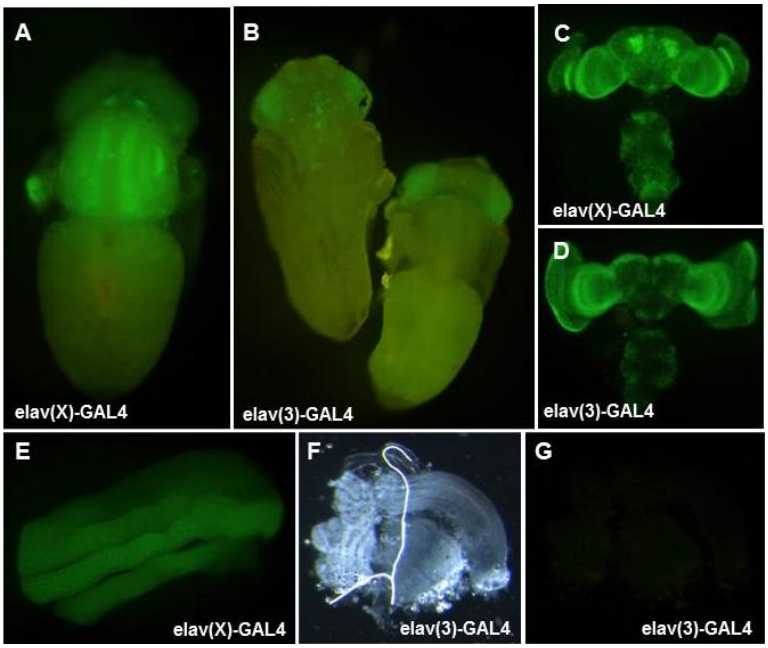

Figure 5.16. GFP expression in pupae and dissected pupal tissues driven by elav-GAL4 drivers. A) Pupa positioned anterior up (dorsal view). B) Pupae positioned anterior up, ventral and dorsal view. C) and D) Brain and ventral nerve cord. E) Dissected thoracic muscle. Dissected thoracic muscle is pictured under F) visible light and G) fluorescence illumination.

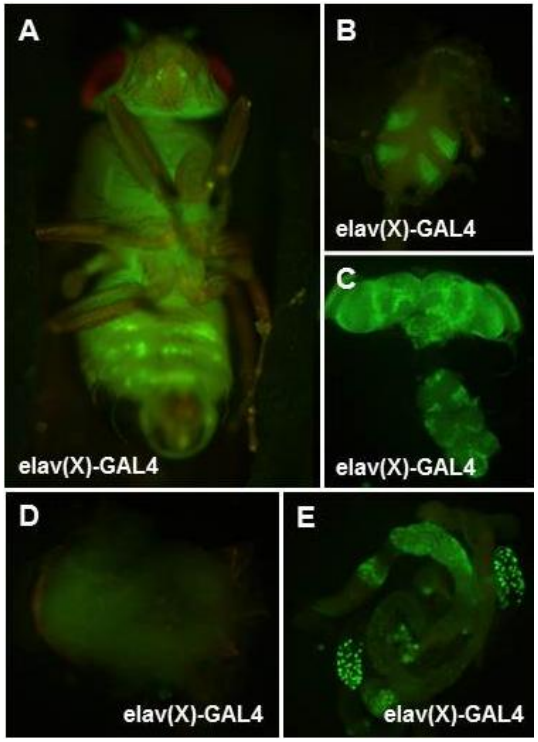

Figure 5.17. GFP expression in adult fly and dissected tissues driven by elav(X)-GAL4. A) 1 day old adult male fly positioned anterior up, ventral view. B) Rectum with GFP expression in rectal papilla cells. C) Brain and ventral nerve cord. D) Thoracic muscles attached to cuticle. E) Testis and gut.

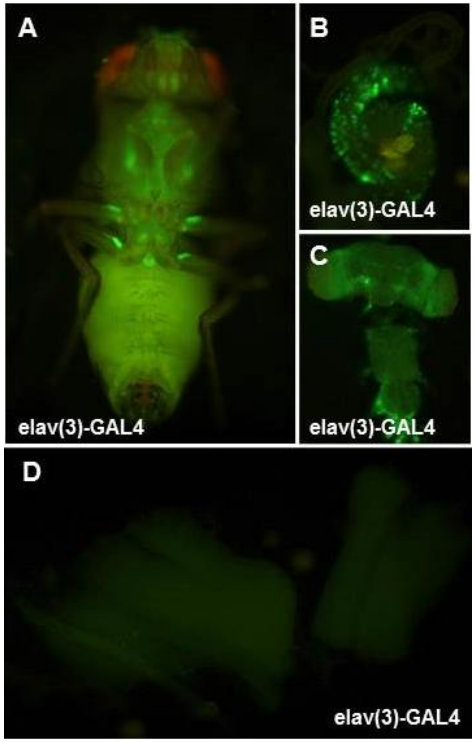

Figure 5.18. GFP expression in adult fly and dissected tissues driven by elav(3)-GAL4. A) 1 day old adult male fly expressing UAS-mCD8-GFP positioned anterior up, ventral view. B) Testis and gut. C) Brain and ventral nerve cord. D) Thoracic muscle fibers.

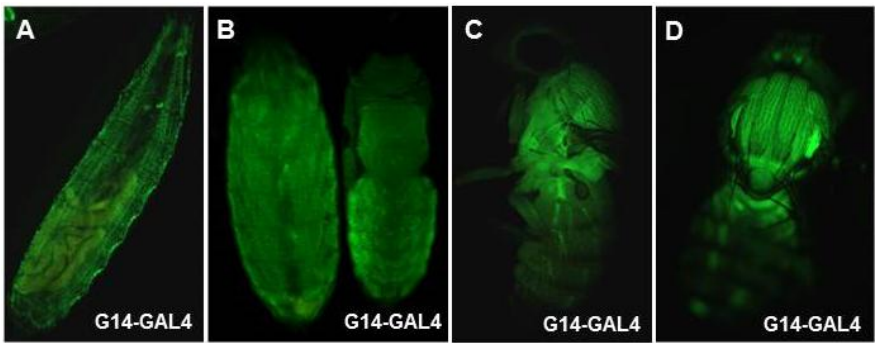

Figure 5.19. G14-GAL4 driven muscle specific expression of GFP in different stages of fly development. A) First instar larva positioned anterior up. Fly food in the gut is autofluorescent. B) Early and late stage pupae positioned anterior up, dorsal view. C) Adult fly positioned anterior up, lateral view. D) Adult fly expressing UAS-mCD8-GFP positioned anterior up, dorsal view. Pictures courtesy of Tea Tuomela.

6. DISCUSSION

6.1 Knocking down COX subunits with neural specific elav-GAL4 drivers produces variable phenotypes

Three nervous-system specific GAL4 drivers were here used to drive RNAi against two COX subunits (IV and Vb) in the fruit fly *Drosophila melanogaster*. The aim was to see if any phenotypes would emerge due to the knockdown and whether these phenotypes could be rescued with AOX coexpression.

The results showed that knocking down COX subunits with neural specific drivers can produce variable phenotypes, including full and partial lethality, locomotor disabilities and decrease in viability. The phenotypes produced due to knocking down COX IV were stronger than knocking down COX Vb, and the phenotypes were also stronger in males than in females. AOX coexpression was able partially to rescue the deleterious phenotypes, whereas Ndi1 coexpression made them worse. Two of the nervous-system specific drivers were shown to give substantial expression in the muscle, which is of great importance considering the aim and purpose of this research.

6.1.1 Neuronal COX knockdown phenotype resembles muscle-specific COX knockdown phenotype

Immobility and poor motor functions were found in flies with COX IV knockdown driven with elav(3)-GAL4 and flies with COX Vb knockdown driven with elav(X)-GAL4. Although these phenotypic characteristics were not quantitated, they were clearly identified in the survival experiment. In order to quantify the disability of locomotor action, a set of climbing tests (described in Fernandez-Ayala *et al*, 2009) should be performed on knockdown flies. Additionally, bang-sensitivity tests (described in Toivonen *et al*, 2001) could be done to verify the damage caused by the neuronal specific knockdown.

A similar type of movement dysfunction was found in flies with muscle-specific COX knockdown (Kemppainen *et al*, unpublished). In the latter experiment, COX knockdown was driven with the muscle-specific G14-GAL4 driver, which resulted in a phenotype of lethality at eclosion and locomotor dysfunction (observed as a decreased ability of the flies to climb the wall of the vial). In this study, the neuronal GAL4 drivers were found to cause leaky expression in the thoracic muscles of the flies in pupal and adult stage (discussed in section 6.1.2). Therefore it cannot be concluded

that the phenotype observed in flies with neuronal-specific COX knockdown, was due to effects solely in the neurons. The experiments carried out do not show if the phenotype resembling that caused by muscle-specific COX knockdown was due to inactivation or death of motor neurons or due to direct dysfunction of the muscle. Shaking and motor dysfunction are symptoms of many common neuronal diseases in humans, for example Parkinson's disease (Dauer and Przedborski, 2003) and multiple sclerosis (MS) (Compston and Coles, 2002), whose pathology is believed to be related to mitochondrial function (Winklhofer and Haass, 2010; Campbell *et al*, 2011). In order to visualize the state of neuromuscular junctions in COX knockdown flies an experiment where membrane-targeted UAS-GFP ("mCD8", Lee and Luo, 1999) expression, driven by the elav-GAL4 drivers, could be imaged. Thus it could prove if the neurons are really damaged.

6.1.2 elav-GAL4 driver leakage complicates interpretation of the results

The distribution of the elav protein (*e*mbryonic *l*ethal *a*bnormal *v*isual system) was first characterized in a study by Robinow and White (1991), in which they saw elav to be produced only in post-mitotic neurons. Since then it has been widely used as a neuronal marker (Yao and White, 1994). The *elav* gene promoter has also been used to construct many *Drosophila* lines expressing GAL4 specifically in neurons. The *elav* gene was later shown to be expressed also in glial cells of *Drosophila* nervous tissue, at least in the embryonic stage (Berger *et al*, 2007).

In this study, elav-GAL4 drivers were shown to be giving expression in muscle, which has not been previously reported. The signal produced by elav-driven expression of GFP varied within the muscles, which could be caused by differential proteolysis of GFP in the dissected tissues. It is also possible that elav-GAL4 drives the expression of transgenes at a lower level in later stages of fly development. The GFP detected in muscle might originate from axons connecting into the muscle fibers of the fly. However, as was previously stated, the UAS-GFP construct used was nuclear-targeted, so the axons should not harbour any GFP. There was also a high expression of GFP in salivary glands of larvae and adult *Drosophila*, which is possibly due to a salivary gland specific enhancer element in the pGawB construct (Gerlitz *et al*, 2002) that was used to make the elav-GAL4 driver line.

Elav-GAL4 drivers have been used by researchers to study and create different disease models in *Drosophila*, for example a model of restless legs syndrome (Freeman *et al*, 2012) and Huntington's disease (Bodai *et al*, 2012). Many of the models are for neurological diseases that include locomotor dysfunction as symptoms. When such models are created using elav-GAL4 drivers, it is

possible that some phenotypic characteristics are due to expression of a target UAS-transgene in muscle. For example, in another study of Huntington's disease (Agrawal *et al*, 2005), the X-chromosomal elav-GAL4 driver was used to drive the expression of mutant human huntingtin. The expression of this protein leads to developmental lethality. The data in the study does not show to which stage the flies developed before death, i.e. it does not show if the flies were dying as larvae or as pupae. If many flies were dying at eclosion, unable to emerge from the puparium, it is possible that the aggregation of mutant huntingtin was also affecting the muscle tissue. Therefore, it must be noted, from this point on, that knockdown using elav-GAL4 drivers must be considered as potentially effective in both nervous and muscle tissue. Previous experiments using elav-GAL4 drivers should be interpreted with care, bearing in mind that the drivers are not exclusively neuronal.

6.1.3 Phenotypes were not detected in Nrv2-GAL4 driven COX knockdown flies: probable late-onset effects

The third neuronal driver used in this study (Nrv2-GAL4) did not produce any COX knockdown phenotypes in *Drosophila melanogaster*. The Nrv2-GAL4 driver has been shown to efficiently drive the expression of UAS-transgenes specifically in the nervous system (Sun *et al*, 1999) and it has also been used successfully to drive AOX expression in a *Drosophila* model for Parkinson's disease (Fernandez-Ayala *et al*, 2009). Thus it seems peculiar why COX knockdown using the same driver, would fail to generate any phenotype. It is possible that non-intentional selection of weak flies, during the upkeep of the stock used in this study, has led to weaker GAL4 expression.

Nrv2-GAL4 might be the only truly nervous specific driver used in this study, as it was shown that the other two neural specific drivers turned out to be leaking expression in the muscle. In a recent study mitochondrial polymerase γ was knocked down in specific neuronal subpopulations using specific GAL4 drivers (Humphrey *et al*, 2012). Knocking down polymerase γ lead to a loss of many core subunits of respiratory chain complexes that are encoded in the mitochondrial DNA, including COX I, II and III. The same paper also showed that neuronal respiratory chain deficiencies caused locomotor defects and neurodegeneration but only in aged flies. Neurodegenerative disorders in humans also show late-onset (Lin and Beal, 2006). Therefore, the knockdown flies studied in our experiments might have been too young to present any phenotype resulting from the truly neuronal-restricted COX knockdown.

6.2 COX knockdown phenotype is influenced by many variables

6.2.1 Influence of COX subunit knockdown on phenotype reflects its function in COX biogenesis

As stated above, COX IV knockdown yields a stronger phenotype than COX Vb knockdown, regardless of the sex of the flies. According to present knowledge of COX biogenesis, COX IV joins the multisubunit protein complex early on whilst attached to COX Va (Stiburek *et al*, 2005). In a recent study by Fornuskova *et al* (2010), association of COX subunits IV and Va to subunit I were shown to be necessary for further assembly of functional COX. Therefore, if COX IV is being knocked down, the amount of functional COX may well be greatly reduced (depending on the effectiveness of the RNAi construct). This decrease in COX would ultimately lead to the observed, severe phenotype in knockdown flies.

COX subunit Vb is added to the respiratory chain complex at a later stage of assembly, in the formation of the assembly intermediate S3 (Nijtmans *et al*, 1998). The catalytic core of functional COX has already been established before joining subunit Vb to the complex. Therefore knocking down subunit Vb in flies should have less dramatic effects on phenotype than COX IV knockdown. However, Galati *et al* (2009) showed in their study that knocking down COX Vb in mammalian cells reduces the amount of COX subunits IV and Va. This result is contradictory to the result of Glerum and Tzagoloff (1997), who showed that knocking down the yeast homolog of mammalian COX Vb in *Saccaramyches cerevisiae* caused very little decrease in the amount of COX subunits IV and Va. Thus it is yet unclear whether knocking down COX Vb affects the amount of COX IV in mitochondria. In this study, the effect of COX Vb knockdown on the phenotype of *D. melanogaster* was milder than that produced by COX IV knockdown. Therefore, it can be presumed that COX Vb knockdown has a more modest effect on COX activity/assembly.

Diaz *et al* (2006) showed that COX deficiency could also have an effect on levels of complex I. In their study, Cox10 knockout mouse fibroblasts had considerably decreased amounts of complex I present in mitochondria. In another mouse model, with supressed expression of COX IV, levels of complex I were also significantly decreased due to the resultant COX deficiency (Li *et al*, 2007). Taking into account these previous results, it is possible that complex I was also affected in the knockdown of COX in *Drosophila*. Owing that COX IV-KD was more efficient than COX Vb-KD, the level of complex I may well be lower in the COX IV-KD. This could have also influenced the phenotype of COX knockdown flies. However, in a study by Antonicka *et al* (2003), patients with

severely reduced COX activity caused by a Cox10 mutation, had normal levels of complex I and other respiratory complexes. In the nematode *C. elegans*, COX deficiency did not cause decrease in the levels of complex I, but a decrease in the activity of the complex (Suthammarak *et al*, 2009). Thus, the role of COX in complex I assembly and stability is still unclear (Lazarou *et al*, 2009). It would have been interesting to see, how each COX subunit knockdown is affecting other respiratory chain complexes and supercomplex structure. This could be analysed using Blue Native polyacrylamide gel electrophoresis (BN-PAGE) (depicted in McKenzie *et al*, 2007).

A much simpler explanation could be the cause of the difference in phenotype severity in the COX knockdown flies. The level of knockdown achieved with the expression of an RNAi construct is never 100 % (Shan, 2010). Therefore there is always residual expression and therefore synthesis of the target protein left within the knockdown cells. In this study, RNAi against COX IV might simply be more efficient in directing *CoIV*-mRNA to degradation than RNAi against *CoVb*-mRNA. An attempt at quantifying the level of COX knockdown was performed by staining fixed sections of fly brain and muscle with COX specific antibodies, but interpreting the images turned out to be challenging. RT-qPCR is the preferred technique in quantifying mRNA levels in a given sample (Nolan *et al*, 2006), but in this case it was considered too laborious to extract nervous-tissue from flies for this type of analysis.

6.2.2 Stronger knockdown phenotype in male flies could be an implication of dosage compensation or perhaps it is due to differences in female and male fly physiology

Male flies were more affected by the knockdown than female flies according to the findings made in this study. A result that isn't entirely suprising when you consider that female and male flies are very different on several levels. Sexual dimorphism has resulted in very different gene expression in males and females. In a study by Chang *et al* (2011), he and his associates showed that there are more than 1 000 genes that differ in their expression level, and at least the same amount of genes that differ in their expressed isoforms, between female and male flies. This level of differing gene expression ultimately leads to differences between female and male physiology and behaviour. Different physiology between females and males can be due to different endocrine signalling. Males and females secrete hormones in varying amounts that will affect for example, the behaviour and growth of an individual. Hormonal signalling is mainly directed by nervous tissue, and in turn nervous tissue is very much affected by hormonal signalling. It has been shown that female and male central nervous systems respond differently to hormonal signals in rats (Cooke *et al*, 1999)

and in *Drosophila* (Belgacem and Martin, 2006). Therefore, it is no surprise if females and males have different phenotypes in flies whose nervous system is subject to knockdown of COX.

A phenomenon related to sexual dimorphism is dosage compensation of X chromosomal genes. Dosage compensation is a process by which X-chromosomal gene products are produced in equal amounts in organisms where gender is determined by the number of X chromosomes (Charlesworth, 1996). In *Drosophila melanogaster*, where male flies have one X chromosome and females have two, dosage compensation doubles the expression level of male X chromosomal genes (Baker *et al*, 1994). In the case of X-chromosomal elav-GAL4 driven COX knockdown, the difference between the observed female and male phenotypes could be due to dosage compensation. More GAL4 is likely to have been expressed in males than in females, although both had just one copy of the driver gene. GAL4 results in active transcription of UAS-RNAi genes. With more GAL4 present in male neurons, the male flies had a stronger knockdown phenotype than females. To test the hypothesis of whether dosage compensation leads to more RNAi in male flies, the expression level of COX subunits should be measured in both male and female flies with elav(X)-GAL4 driver.

Dosage compensation is unlikely to account for the difference in knockdown phenotype between male and female flies with elav(3)-GAL4 driven COX knockdown however. This driver is situated in chromosome 3 and therefore both sexes should produce GAL4 in equal amounts. The same pattern of difference between COX knockdown phenotypes in male and female flies has been observed in other studies as well (Ni *et al*, 2007). Ni and his colleagues proposed that a difference in UAS-RNAi expression could be due to the difference in the developmental time of male and female flies. Because male flies take slightly more time to developed, they may well produce more RNAi hairpin molecules. However, UAS-RNAi expression is entirely dependent on the GAL4 driver and therefore the difference in UAS-RNAi expression reflects differences in GAL4 expression between male and female flies. The reason behind the difference in male and female phenotypes cannot completely rule out dosage compensation, as the RNAi constructs contain X chromosome derived elements (Ni *et al*, 2007). These elements could recruit proteins required for dosage compensation in male flies, even though the construct is not actually situated in X chromosome.

6.3 Addition of alternative respiratory chain components changes redox homeostasis in mitochondria of COX knockdown flies

6.3.1 AOX alleviates respiratory chain deficiency related symptoms by restoring redox balance

Alternative oxidase from the tunicate *Ciona intestinalis* has been designed to be expressed constitutively in human cells (Hakkaart *et al*, 2006) and conditionally in *Drosophila melanogaster* (Fernandez-Ayala *et al*, 2009). In a more recent study, AOX has been expressed in a whole mouse model (El-Khoury *et al*, 2013). In all of the above cases, AOX expression does not have any detrimental effects on the organism, and it stays inactive in mitochondria until the cytochrome component of the respiratory chain is blocked, or the redox state of the cell is highly reduced. In this study, AOX expression was able to rescue either partially, or fully, the phenotypes resulting from knockdown of COX subunit IV and Vb, respectively. In previous studies, AOX expression has been used to rescue other types of mitochondrial deficiency, including Parkinson's disease phenotype induced by *dj-1β* mutation in *Drosophila* (Fernandez-Ayala *et al*, 2009), dopaminergic cell loss in flies with knockdown of mitochondrial polymerase γ catalytic subunit (Humphrey *et al*, 2012) and decreased cell respiration in Cox15 depleted human skin fibroblasts (Dassa *et al*, 2009a).

In this study, AOX coexpression was not sufficient to make COX IV knockdown flies survive after the eclosion. It would seem that the rescue capability of AOX is tied to the extent of COX dysfunction (Fernandez-Ayala *et al*, 2009). AOX expression, driven in this study by elav-GAL4 drivers, produced a complete rescue of COX Vb knockdown flies, this is suggestive of COX Vb knockdown having a lesser effect on levels and activity of COX than does COX IV knockdown. The alleviating effect of AOX in mitochondria with COX dysfunction is based on the fact that it can partially bypass the respiration chain and transfer electrons straight from reduced ubiquinol to oxygen (Humphrey *et al*, 2012). Subsequently it also relieves metabolic acidosis and diminishes ROS production in mitochondria (Rustin and Jacobs, 2009). The most significant advantage of AOX expression in a situation of decreased COX activity is its ability to revive ATP production, as electron flow and subsequent proton pumping are allowed to continue through complex I of the respiratory chain (El-Khoury *et al*, 2013). However, ATP production is not fully restored to initial levels, as two of the three proton pumping enzymes are bypassed. In a case of AOX rescue, ATP production is restored to a level over the threshold that allows vital cellular functions to carry on. This is likely to be the situation in COX Vb knockdown flies, where AOX expression rescued all

detrimental phenotypes. In COX IV knockdown flies however, ATP production could not be restored above the required threshold, most likely because of a greater reduction in COX activity.

This study and the previous studies show the capability of AOX to alleviate symptoms caused by mitochondrial (especially COX) dysfunction. Therefore it should be considered as a potential future therapeutic tool to compensate for these deficiencies in humans. It has already been expressed beneficially in human cells (Dassa *et al*, 2009a, 2009b) but there are still a lot of open questions to be answered about the enzyme function in physiological conditions before it can be applied to human therapeutics.

6.3.2 Ndi1 expression exacerbates detrimental phenotypes by feeding additional electrons to the defective respiratory chain

As mentioned before, Ndi1 is a protein composed of a single polypeptide chain that can transfer electrons from NADH to ubiquinone thus bypassing complex I of the respiratory chain (Cho *et al*, 2012). Ndi1 expression has been previously shown to prolong life expectancy in flies (Bahadorani *et al*, 2010; Sanz *et al*, 2010b) and also rescue flies with complex I deficiency (Cho *et al*, 2012). However, Ndi1 cannot rescue deleterious phenotypes resulting from other respiratory chain complex deficiencies (Humphrey *et al*, 2012). The inability of Ndi1 expression to rescue flies with complex IV deficiency was also documented in this study, with some interesting additional findings. Ndi1 coexpression made flies die even faster than when they were expressing a knockdown construct only. This could be due to the fact that Ndi1 bypasses complex I of the respiratory chain. The overall proton pumping activity of complex I is decreased when Ndi1 transfers electrons from NADH to ubiquinone without any proton translocation. Thus the electrochemical gradient is lower and ATP production is decreased. The even lower ATP levels resulting from Ndi1 expression could cause the early death observed in COX knockdown flies. Sanz *et al* (2010a) showed that ROS production was decreased in aging flies that were expressing Ndi1. However, in this study the electrons fed by Ndi1 into the respiration chain possibly cause an increase in the production of reactive oxygen species, which will bring further damage to the mitochondria. This damage ultimately leads to the observed early death of Ndi1 expressing COX knockdown flies.

6.4 Experimental considerations and future prospects

In addition to new experiments mentioned above (climbing and bang-sensitivity -assays and BN-PAGE or RT-qPCR for quantification of COX knockdown) some of the experiments presented here could be validated by thorough repeats with some moderate changes in the experimental set-up. The lethality assays performed in this study show the number of flies that have eclosed from each cross. Comparison between the number of eclosed knockdown flies and wild-type flies is not the best way to evaluate the effect of knockdown on lethality, because it is affected by the overall number of eggs laid. Therefore it would be more informative if the number of eclosed flies would be presented as percentages of the number of eggs laid. In order to do so, either the number of eggs or pupae should be counted, depending on whether the knockdown induces lethality in early or late stage of development, respectively. The number of eggs or pupae could then be normalized using the respective number in wild-type vials. Another approach could be to normalize the number of eclosed flies by transferring a specific amount of eggs, after crossing, into fresh fly food vials (Agrawal *et al*, 2005).

Producing comparable data of the level of COX knockdown from paraffin sections of fly brain and muscle proved difficult using the methods shown above. In fact, sectioning of paraffin embedded fly tissues is a technique rarely used anymore. Most of the immunostaining is currently done on whole mount tissues. This eliminates many steps of the staining process and reduces the handling time of the tissue. It also gives a better view of the three dimensional structure of the tissue because it does not need to be sectioned. In future experiments, a whole mount immunostaining technique would be used.

7. CONCLUSIONS

The first aim of this study was to characterize the phenotypes of neuron-specific knockdown of COX in *Drosophila melanogaster*. Out of the three different neuronal specific GAL4 drivers that were used to drive RNAi expression in flies, two were able to produce observable phenotypes. The severity of the phenotype seemed to be dependent on the COX subunit, which was being knocked down. COX IV-KD produced stronger phenotype than COX Vb-KD, which is thought to reflect their different roles in COX biogenesis. COX IV is added to the enzyme complex earlier than COX Vb and depleting COX IV would thus be expected to cause more pronounced COX deficiency than COX Vb depletion. The effect of COX subunit knockdown on the integrity of mitochondrial supercomplexes or on the function of other respiratory chain complexes should be studied further.

The second aim of the study was to test whether AOX expression is able to rescue the deleterious phenotypes. The experiments confirmed previous results in that AOX is able to partially or fully rescue phenotypes resulting from COX deficiency. Thus AOX shows its potential to functions as a potential therapeutic agent for COX dysfunction. However, a somewhat surprising result showed that expressing Ndi1 in COX deficient flies makes the phenotype even worse. The ability of alternative respiratory enzymes to alleviate respiratory chain dysfunction logically reflects their ability to restore ATP production. I hypothesise that, in case of AOX expression in COX deficient neurons, proton pumping through complex I is facilitated, which restores ATP production over the threshold level that allows vital cellular pathways to stay active. In case of Ndi1 coexpression, electrons bypass complex I of the inhibited respiratory chain, which subsequently drops the level of ATP production even further, leading to the observed more severe phenotypes.

This study was also able to show the permissive expression of neuronal GAL4 drivers. A more precise examination of the elav-GAL4 drivers expression pattern showed leaky expression in the muscle of pupal and adult stage flies. Therefore it cannot be concluded that the observed phenotypes were exclusively due to neuronal knockdown of COX. This also has implications for the interpretation of other experiments that utilize these GAL4 drivers.

According to the results presented here and in previous studies, AOX should be considered as a potential therapeutic agent for patients with COX deficiency. However, more studies on the function of AOX in mammalian cells are still required. Also the development of safe and applicable vectors for gene therapy is not yet advanced and the use of AOX in the clinic is therefore still far in the future.

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APPENDICES

Appendix 1.

Crossing schemes to study the effects of COX subunit knockdown on viability and phenotype of *Drosophila melanogaster* and the attempt to rescue deleterious phenotypes with AOX coexpression, and to characterize neural specific elav-GAL4 drivers expression pattern.

A Following crosses were made to study the effects of COX subunit knockdown on development (lethality assay):

i. $\frac{UAS\text{-}RNAi}{UAS\text{-}RNAi}\ virgins \times \frac{Nrv2\text{-}GAL4}{CyO}$

ii. $\frac{UAS\text{-}RNAi}{UAS\text{-}RNAi}\ virgins \times \frac{elav(3)\text{-}GAL4}{Sb}$

iii. $\frac{UAS\text{-}RNAi}{UAS\text{-}RNAi}\ virgins \times \frac{elav(X)\text{-}GAL4}{y}$

B Following crosses were made to determine if AOX is able to rescue the detrimental effects of COX subunit knockdown on development (lethality assay):

i. $\frac{UAS\text{-}RNAi}{UAS\text{-}RNAi};\frac{UAS\text{-}transgene}{UAS\text{-}transgene}\ virgins \times \frac{elav(3)\text{-}GAL4}{elav(3)\text{-}GAL4}$

ii. $\frac{UAS\text{-}RNAi}{UAS\text{-}RNAi};\frac{UAS\text{-}transgene}{UAS\text{-}transgene}\ virgins \times \frac{elav(X)\text{-}GAL4}{y}$

C The following crosses were set up to study the effect of COX subunit knockdown on survival:

i. $\frac{UAS\text{-}RNAi}{UAS\text{-}RNAi}\ virgins \times \frac{Nrv2\text{-}GAL4}{Nrv2\text{-}GAL4}$

ii. $\frac{UAS\text{-}RNAi}{UAS\text{-}RNAi}\ virgins \times \frac{elav(3)\text{-}GAL4}{elav(3)\text{-}GAL4}$

iii. $\frac{elav(X)\text{-}GAL4}{elav(X)\text{-}GAL4}\ virgins \times \frac{UAS\text{-}RNAi}{UAS\text{-}RNAi}$

D To study if AOX expression is capable of rescuing decreased viability in *Drosophila melanogaster*, following crosses were set up:

i. $\frac{UAS\text{-}RNAi}{UAS\text{-}RNAi};\frac{UAS\text{-}transgene}{UAS\text{-}transgene}\ virgins \times \frac{elav(3)\text{-}GAL4}{elav(3)\text{-}GAL4}$

ii. $\frac{elav(X)\text{-}GAL4}{elav(X)\text{-}GAL4}\ virgins \times \frac{UAS\text{-}RNAi}{UAS\text{-}RNAi};\frac{UAS\text{-}transgene}{UAS\text{-}transgene}$

E The following crosses were done to characterize neural specific elav-GAL4 drivers expression pattern:

i. $\frac{elav(3)\text{-}GAL4}{elav(3)\text{-}GAL4}\ virgins \times \frac{UAS\text{-}GFP}{UAS\text{-}GFP}$

ii. $\frac{elav(X)\text{-}GAL4}{elav(X)\text{-}GAL4}\ virgins \times \frac{UAS\text{-}GFP}{UAS\text{-}GFP}$

Appendix 2.

Representative slides of H&E stained fly brain is presented in Figure A2.1. COX knockdown flies stuck in fly food and flies unable to eclose are presented in Figure A2.2.

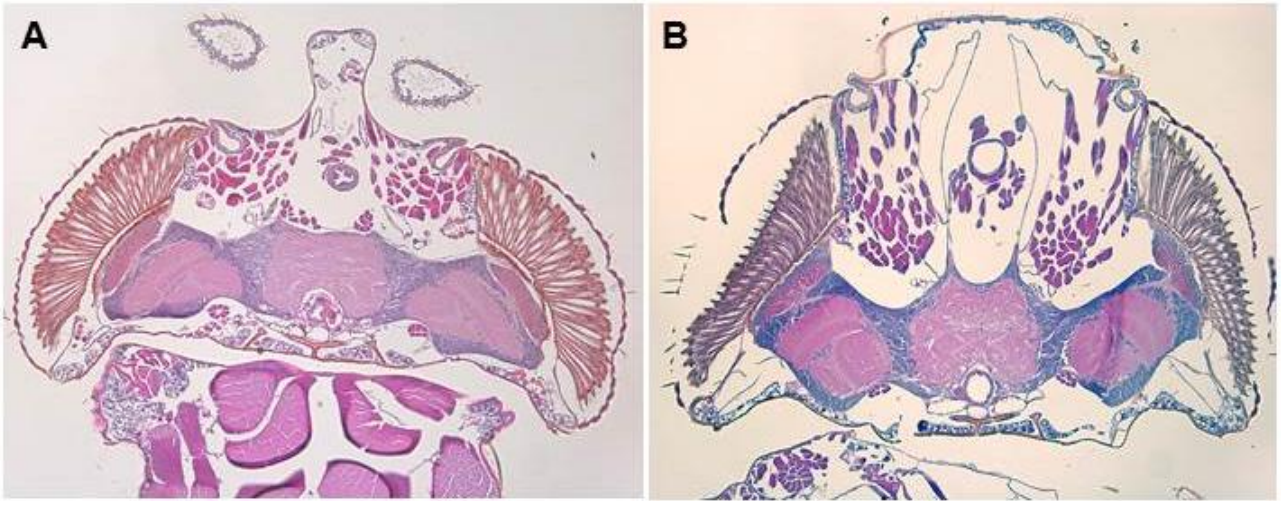

Figure A2.1. HE stained paraffin sections of COX subunit IV knockdown fly brain when knockdown is driven with elav(X)-GAL4. A) A representative slice of COX IV knockdown female brain. B) A representative slice of wild-type female brain.

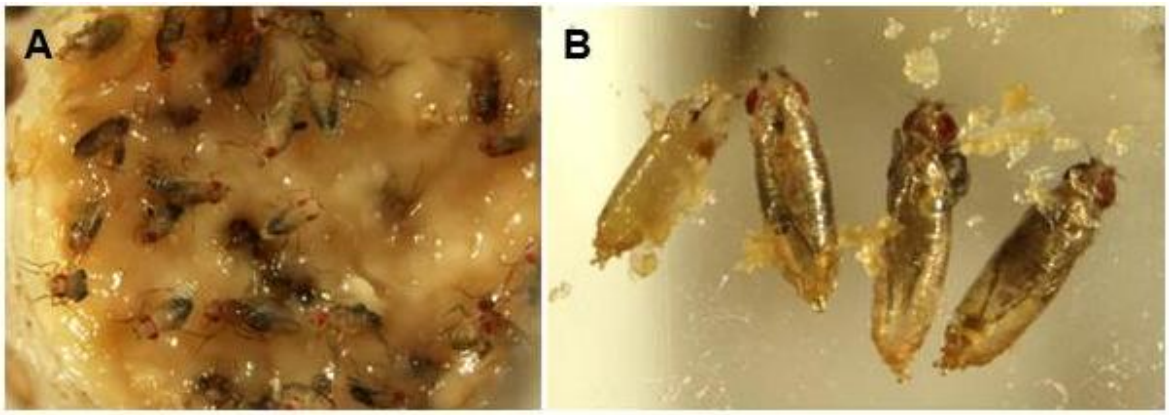

Figure A2.2. Influence of COX IV knockdown on fly phenotype when knockdown is driven with elav(X)-GAL4. A) COX IV;AOX flies all get stuck in the food and die soon after eclosion. B) COX IV knockdown flies cannot even eclose and die in the pupal case.

Appendix 3.

Lethality assay was also performed on flies with COX subunit Vb knockdown and GFP expression driven by elav(X)-GAL4. GFP was expressed as a control for AOX. The test was repeated twice with same type of results (Figure A3).

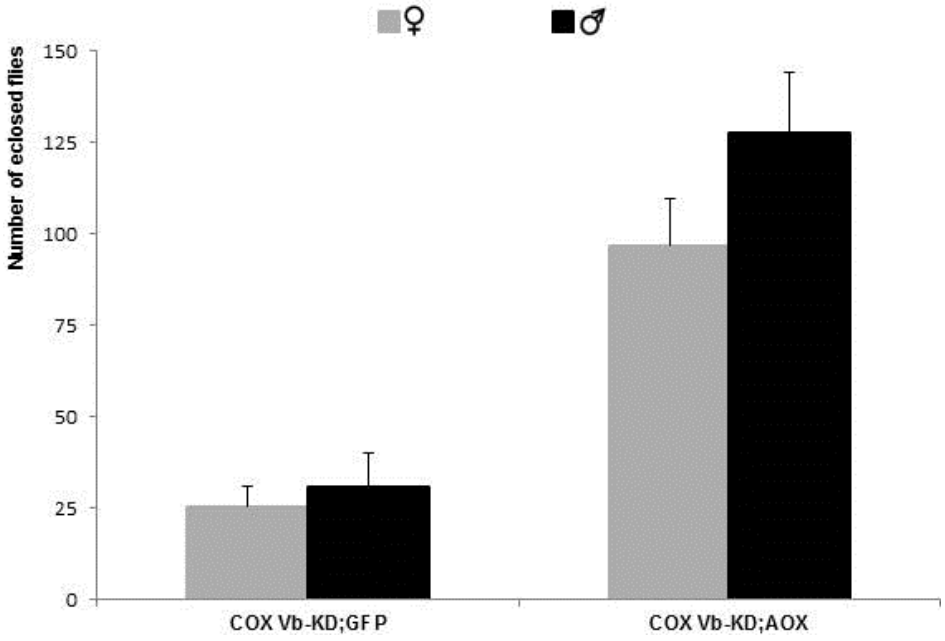

Figure A3. Mean number of eclosed flies when COX subunit Vb knockdown and transgene expression is driven with elav(X)-GAL4. Females are expressing the transgene and RNAi, whereas male flies are not. Means are presented ± SD.

Appendix 4.

Images of COX subunit knockdown in brain and muscle stained with antibodies are presented in Figure A4.1 and A4.2. Antibody against COX IV was used to stain COX in selected tissues, because it was proven functional by previous work (even though it is one of the subunits knocked down).

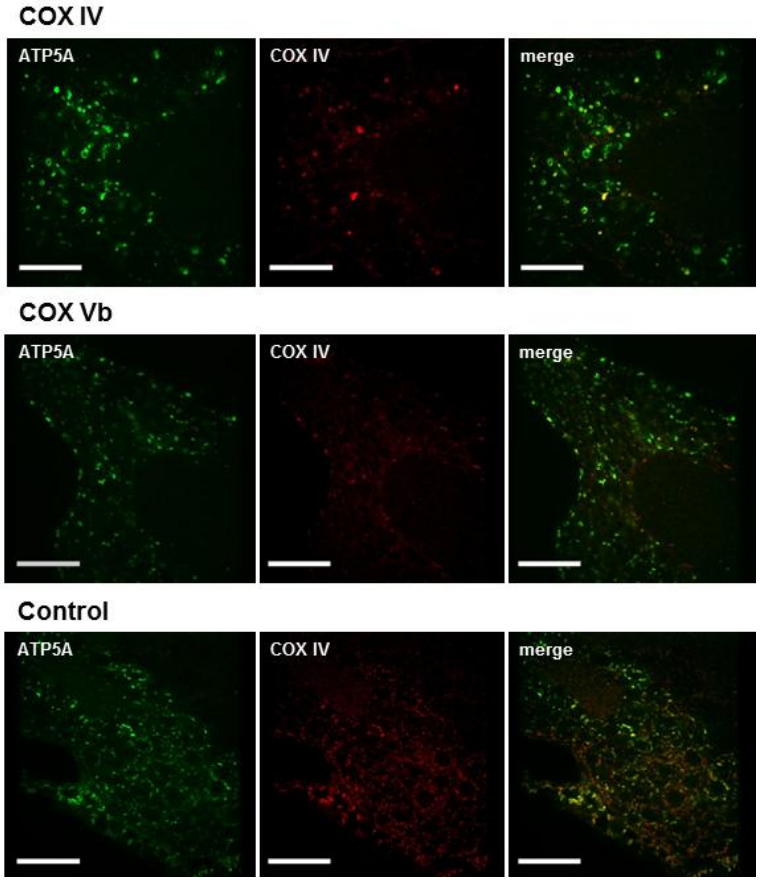

Figure A4.1. COX knockdown in the central nervous system of *Drosophila melanogaster*. COX subunit knockdown is driven with elav(X)-GAL4. ATP synthase subunit 5 (ATP5A) is a control and should not be affected by the COX subunit knockdown. Scale bar 20 µm.

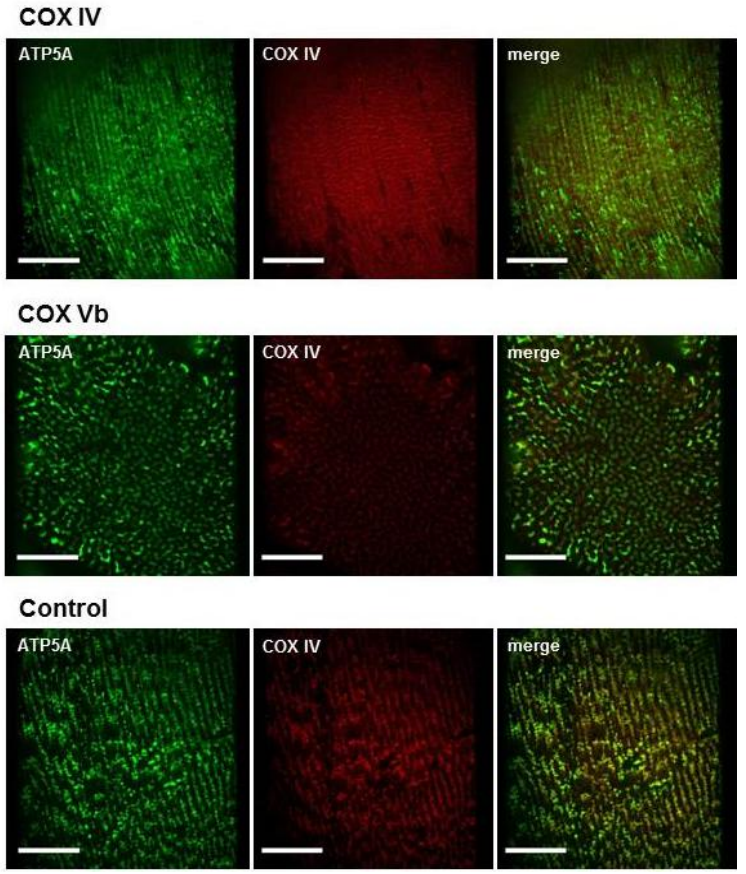

Figure A4.2. COX knockdown in the muscle tissue of *Drosophila melanogaster*. COX subunit knockdown is driven with elav(X)-GAL4. ATP5A is a control and should not be affected by the COX subunit knockdown. Scale bar 20 µm.